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THE NUCLEAR ROLE IN THE ULTRAVIOLET INACTIVATION OF NEUROSPORA CONIDIA[1]

AMOS NORMAN

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THREE FIGURES

INTRODUCTION

An analysis of the survival curves of ultraviolet irradiated Neurospora conidia indicates that the inactivation of the conidia is a consequence of the inactivation of the nuclei (Norman, '51). The inactivation cross section per nucleus is about twice as large in uninucleate (haploid) conidia as in multinucleate conidia; the smaller cross section per nucleus in the multinucleate conidia is apparently due to some sort of nuclear interaction. The present study of the influence of nuclear number on the inactivation and photoreactivation of Neurospora conidia was undertaken to establish more firmly the role of the nucleus in the inactivation process, and to learn something more of the nature of the nuclear interaction in multinucleate conidia.

MATERIALS AND METHODS

Neurospora crassa 1A, which gives multinucleate macroconidia, and the mutant strain pe^{m}Fl, which gives uninucleate microconidia were grown on minimal medium (Norman, '51). On this medium the macroconidia average 2.3 nuclei per conidium. This number is increased to 4.2 and 5.9 by adding,

[1] This work was done in the Department of Zoology, Columbia University, New York; it was made possible by an A. E. C. Postdoctoral Fellowship administered by the National Research Council, and in part by grants from the United States Public Health Service and the American Cancer Society.

respectively, ½% and 1% of Bacto casamino acids to the minimal medium (Huebschman, '52).

Saline suspensions of $10^2 - 10^3$ conidia per milliliter were irradiated in open beakers by a sterilamp whose energy output is predominantly (85%) in the 254 mμ mercury resonance line. During irradiation the conidia were stirred constantly. Photoreactivation was accomplished by the light of an H–5 mercury lamp filtered by a copper sulfate solution. During photoreactivation the conidial suspensions were vigorously air cooled. The ability to form colonies in a minimal sorbose medium (Norman, '51) was used as the criterion for the viability of the conidia.

In order to count the nuclei the conidia were fixed in Carnoy's, hydrolyzed for 12 minutes at 60°C. in 1N HCl, and stained in a solution containing two parts of 1% Azure A, one part of 10% sodium bisulfite, and one part of 1N HCl.

RESULTS

The interpretation of the survival curves is based on the following two assumptions: (A) a conidium will survive if at least one of its nuclei survives; (B) the probability, S, that a nucleus survives a dose, D, of radiation is given by:

$$S = e^{-kD} \qquad (1)$$

where k, the inactivation cross section per nucleus, is independent of D. From these assumptions it follows (Atwood and Norman, '49) that at sufficiently high dose the fraction of conidia, S(n), surviving a dose, D, is given by:

$$S(n) = \bar{n}\, e^{-kD} \qquad (2)$$

where $\bar{n}$ is the average number of nuclei per conidium. A plot of log S(n) against D should give, at high dose, a straight line of slope k which extrapolates back to give, at D = 0, the value of $\bar{n}$, the average number of nuclei per conidium.

In figure 1 are shown the nuclear distributions in conidia of the macroconidial strain when grown on three different media. In figure 2 are shown the survival curves for these

conidia and for the uninucleate microconidia. It can be seen that the values of $\bar{n}$, determined by extrapolating back the linear portion of the survival curves to zero dose, are in good agreement with the average number of nuclei per conidium, determined by direct count of the nuclei. It can also be seen that the inactivation cross section per nucleus, k, is about twice

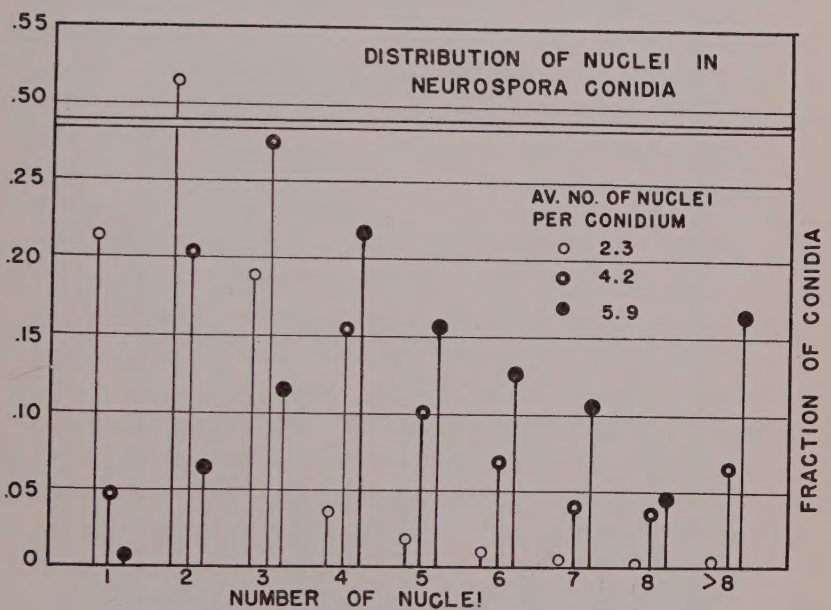

Fig. 1 Distribution of nuclei among the conidia of *Neurospora crassa* 1A. The distributions are based on counts of at least 500 conidia.

as large in the uninucleate microconidia as in the multinucleate macroconidia.

Equation (2) is derived under the assumption that k is independent of the number of nuclei per conidium. The analysis of the survival curves of the macroconidia by means of equation (2) becomes suspect, therefore, if the uninucleate and multinucleate components of the macroconidia exhibit different values of k. A glance at figure 1, however, shows that the uninucleate component is significant only in the conidial population with an average of 2.3 nuclei per conidium. In this

population if the uninucleate component has twice the inactivation cross section per nucleus, k, of the multinucleate component, then the linear portion of the survival curve will continue to exhibit a slope, k, characteristic of the multinucleate component; however, the value of $\bar{n}$, determined by

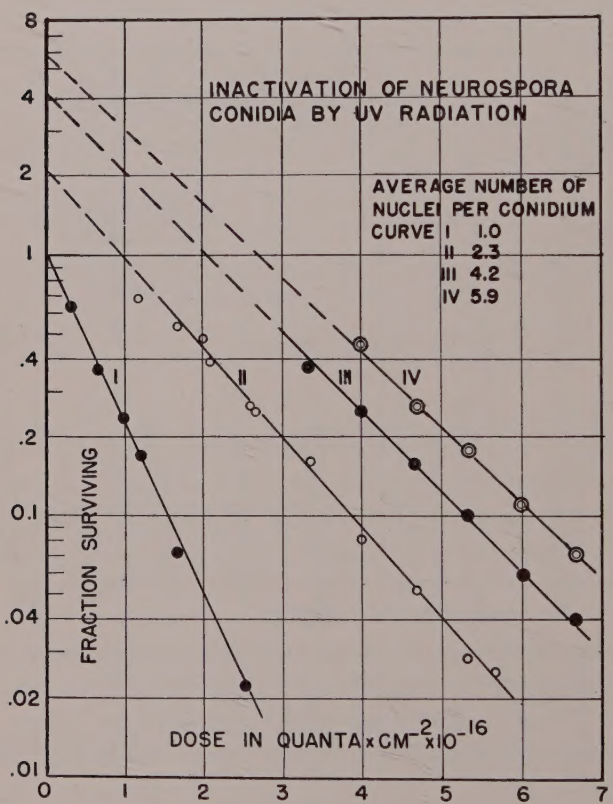

Fig. 2 The inactivation of *Neurospora* conidia by ultraviolet radiation. The wavelength is 254 mμ.

extrapolating back the linear portion of the survival curve, will be lowered about 10% below the value of the actual average number of nuclei per conidium. A statistical analysis of the data indicates that $\bar{n}$, determined from the survival curve, is significantly lower in this population than the aver-

age number of nuclei per conidium, determined by direct count. The value of $\bar{n}$, moreover, is lower by about 10% than the value of the average nuclear number (Norman, '51).

The agreement between the observed and predicted lowering of the value of $\bar{n}$ supports the major premise, namely, that the uninucleate component of the macroconidia has about twice the inactivation cross section per nucleus of the multinucleate component. This, in turn, implies that the difference in slope between curve 1 and the other three curves in figure 2 is not due to some special difference between the micro- and macroconidial strains, but is due to some difference between uninucleate (haploid) and multinucleate conidia in general.

With ultraviolet radiation, however, shielding must be ruled out as a factor in the apparent difference of sensitivity of the micro– and macroconidia. This has been done by a measurement of the transmission of the two types of conidia (Norman, '51). Less direct evidence that the size difference between micro– and macroconidia does not alter the nuclear shielding significantly is furnished by the observation that in multinucleate conidia the inactivation cross section per nucleus remains constant as the average number of nuclei per conidium is increased from 2.3 to 5.9, although the conidial volume also increases, roughly, as the number of nuclei. It seems, therefore, that the decrease in the inactivation cross section per nucleus in the multinucleate conidia is due to some kind of nuclear interaction.

If the assumption, (A), that a conidium will survive if at least one of its nuclei survives is valid, it should be necessary to reactivate only a single nucleus per conidium. Furthermore, since the probability that a nucleus will not be inactivated is related to the inactivating dose by equation (1), we expect a similar relation to hold between the probability that a nucleus is not photoreactivated and the dose of reactivaing light. From this it follows that the number of conidia, c, reactivated by a dose, D, is given by:

$$c = C\,(1-e^{-rD}) \qquad (3)$$

where C is the maximum possible number of photoreactivated conidia, and r is the photoreactivation cross section *per conidium*. Clearly, the larger the number of nuclei capable of photoreactivation per conidium the larger the magnitude of r. The photoreactivation of a given conidial population, therefore, will be described by equation (3) only if the population is homogeneous with respect to the number per conidium of nuclei having the potentiality of being reactivated.

From the survival curves of the macroconidia with and without photoreactivation we can compute the corresponding values of the nuclear inactivation cross sections, k (Norman, '51). From these values of k and the equation (1) we can then compute the probability, at any dose of ultraviolet radiation, that it is possible to photoreactivate an inactivated nucleus. This probability, together with the nuclear distributions given in figure 1, leads to the distributions of nuclei which can be reactivated in the various conidial populations. The photoreactivation experiments were done for ultraviolet doses corresponding to conidial survivals of from 5% to 30%; at these doses the fraction of inactivated conidia with more than one nucleus capable of reactivation is less than 15%. Because of the rather large scatter observed in the reactivation experiments this fraction is not significant and the populations of inactivated conidia may be considered to contain only a single nucleus that can be reactivated per conidium. The photoreactivation of the conidia should be described, then, by equation (3), and r becomes, presumably, the photoreactivation cross section *per nucleus*.

In figure 3 are given the plots of log $(1 - c/C)$ vs. D which, according to equation (3), should be straight lines of slope r. As can be seen from the figure, straight lines are obtained for each of the stocks used; the slopes, r, of these lines do not differ significantly for the three conidial populations used which average 1.0, 2.3, and 4.2 nuclei per conidium. Moreover, r is apparently independent of the ultraviolet dose over the range of dose used. This range corresponds to from one to eight "hits" per nucleus where a "hit" is defined as the

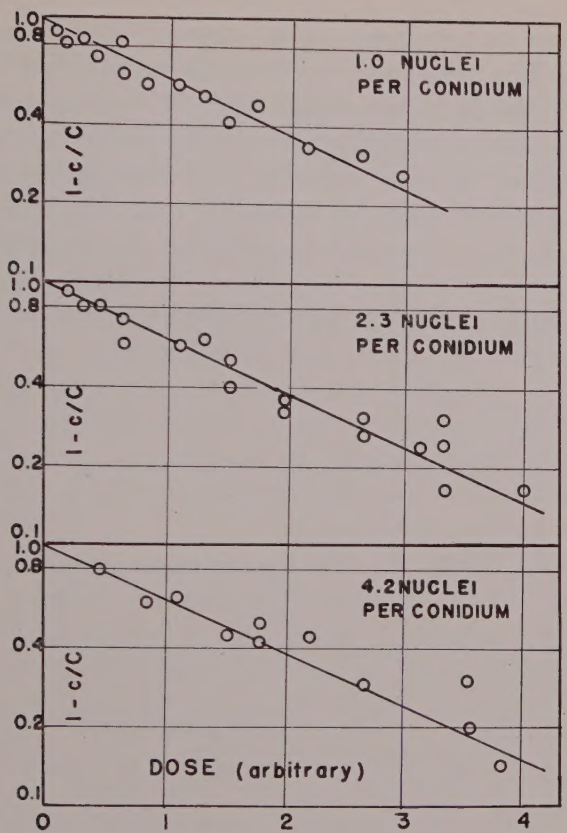

Fig. 3 The photoreactivation of *Neurospora* conidia. Each curve was run for ultraviolet doses corresponding to conidial survivals of from 5 to 30%. C is the maximum number of reactivable conidia; c is the number of conidia reactivated at a given dose of reactivating light.

dose corresponding to a 37% survival of the uninucleate conidia (in the dark).

DISCUSSION

Equation (2) is derivable under the assumption that the inactivation of an organism is the consequence of the inactivation of certain units within it, and that these units are inactivated according to equation (1). The two parameters, $\bar{n}$ and k, then, refer to the average number of these units per

organism and to the inactivation cross section per unit, respectively. The description of a given survival curve by equation (2) leads to the assignment of values to these two parameters. The survival curves of Neurospora conidia, inactivated by ultraviolet radiation, are adequately described, at sufficiently high dose, by equation (2). The value of the parameter, $\bar{n}$, is in close agreement with the average number of nuclei per conidium determined by direct count. This agreement holds as the average number of nuclei per conidium is varied from 1.0 to 5.9; and it holds when several other ultraviolet wavelengths are used to inactivate the conidia (Norman, '51). It seems reasonable, therefore, to assume that the units concerned in the inactivation process are the nuclei, and that the conidial inactivation is a consequence, thus, of the nuclear inactivation.

Calculations based on the inactivation cross section per nucleus and on the nuclear distributions indicate that essentially every conidium which can be photoreactivated contains only one nucleus capable of reactivation. We expect, therefore, that the various conidial populations will exhibit the same kinetics of photoreactivation. This expectation is born out by the data (fig. 3). To this extent, at least, the photoreactivation data support the assumption that the nuclei play the central role in the ultraviolet inactivation and in the photoreactivation of Neurospora conidia.

Atwood (unpublished data) has shown that recessive lethal mutations contribute negligibly to the ultraviolet inactivation of multinucleate conidia. On the other hand, the frequency of lethal mutations is high enough to contribute to the inactivation of the uninucleate (haploid) conidia where, of course, every lethal is expressed. It has been suggested, therefore, that the difference in the inactivation cross sections per nucleus in the multinucleate and uninucleate conidia is due to the expression of lethal mutations in the latter (Norman, '51). On this hypothesis the nuclear interaction leads to the suppression of the expression of (recessive) lethal mutations

in the multinucleate conidia. If the hypothesis is correct, then the mutation frequency vs. ultraviolet dose curve (Atwood, unpublished data) indicates that the production of lethal mutations and of the nongenetic lethal effects that are responsible for the inactivation of the multinucleate conidia are not independent; i.e., the nuclear interaction may have some effect on the mutation production as well as on its expression. At any rate, whatever the nature of the nuclear interaction, the data in figure 2 indicate that it is fully expressed in conidia containing two nuclei; the presence of more than two nuclei does not result in a further decrease of the inactivation cross section per nucleus. From figure 3 it can be seen that the interaction has no significant effect on the photoreactivation cross section per nucleus.

Finally, it is interesting to note certain similarities between the data of figure 2 and the data of Luria and Dulbecco ('49) and Dulbecco ('52) concerning multiplicity reactivation in bacteriophage. They observe, essentially, a smaller inactivation cross section per phage particle in multiply infected bacteria than in singly infected bacteria. Their curves, thus, are quite similar to those in figure 2, with the average number of nuclei per conidium replaced, of course, by the average multiplicity of infection. It is possible that these similarities reflect a common mechanism of recovery from ultraviolet radiation damage in Neurospora and bacteriophage.

SUMMARY

The inactivation of Neurospora conidia by ultraviolet radiation can be accounted for on the assumption that the conidial inactivation is a consequence of the nuclear inactivation. The inactivation cross section per nucleus is about twice as large in uninucleate as in multinucleate conidia. The kinetics of photoreactivation is consistent with the assumption that the reactivation of the conidia is the consequence of the reactivation of a single nucleus per conidium.

ACKNOWLEDGMENTS

I should like to express my gratitude to Dr. K. C. Atwood for innumerable stimulating ideas, some of which are incorporated in this work. I should like to thank Miss Rachel McMaster for help with the cytological work, and to acknowledge the aid of the late C. Huebschman in pointing out the influence of growth medium on the number of nuclei per conidium.

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ANALOGUES OF NUCLEIC ACID DERIVATIVES AND THE GROWTH AND DEVELOPMENT OF RANA PIPIENS

1. THE CYCLIC INHIBITION OF THE DEVELOPMENT OF RANA PIPIENS[1]

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ELEVEN FIGURES

The intimate relationship between nucleic acids and the fundamental processes of growth and differentiation has been a matter of speculation and investigation for many years. With the recognition of structural analogues as a biochemical tool a whole new approach to the problem of nucleic acid synthesis was made possible. Test systems were devised in which the activity of these antimetabolites inhibiting some biochemical process could be readily observed.

Hitchings and his collaborators ('50a, '50b) have made an intensive study of the inhibition of the growth of *Lactobacillus casei* and other systems by analogues of purine and pyrimidine bases. Over a period of 10 years several hundred such analogues were synthesized and tested. They were found to have a variety of biological effects which were attributed to various types and degrees of interference with biosynthesis of nucleic acids and related compounds. The desirability of testing such antimetabolites on a developing form is obvious. Such studies might contribute to the comparative biochemistry of nucleic acid metabolism and might provide some

[1] The major portion of this report was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at New York University.

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knowledge as to the relationship of specific compounds to morphologic development. The amphibian egg lends itself readily to experimental and biochemical observations on early morphogenesis. The chick embryo is not easily accessible for chemical studies before the second or third day of incubation, by which time early morphogenesis is ended.

It was the consideration of the preceding facts that led to undertaking the following study of antimetabolites and amphibian development. A preliminary attempt at such studies (Bieber, Nigrelli and Hitchings, '52) revealed that despite the difficulties inherent in immersion techniques careful experimental manipulation makes the approach feasible. Along with the purine and pyrimidine analogues, aminopterin (4-aminopteroylglutamic acid) was used as a reference compound. The action of aminopterin in interfering with nucleic acid synthesis has been established (Goldthwait and Bendich, '51; Skipper et al., '50). This compound had previously been tested on the developing chick (Wagley and Morgan, '48; Karnofsky et al., '49; Cravens and Snell, '50; Snell and Cravens, '50) and in a preliminary fashion on the developing frog (Rosenbaum and Verlardo, '51).

MATERIAL AND METHODS

Eggs were obtained from female *Rana pipiens* by induced ovulation (Rugh, '34). They were gently stripped into a dry enamel pan and fertilized by spraying them with a sperm suspension made up by macerating one pair of frog testes in 20 cm^3 of spring water. Ninety per cent fertilization was arbitrarily chosen as the minimum requirement for selecting any clotch of eggs for experimental purposes. Following successful fertilization several procedures were employed:

I. All compounds were tested in a preliminary fashion by immersing embryos, chosen at the two cell stage, in different concentrations made up in spring water and ranging from 1 to 50 mg %. Fifty embryos, 25 per finger bowl, were used for each concentration. The bowls were kept in an incubating

refrigerator at 18°C. Readings of developmental progress were made at regular intervals using the Shumway tables (Shumway, '40) as a guide, and by comparison with control groups. The present report will be concerned with development through Shumway stage 25. The most advanced system or structure found in at least 50% of the embryos was taken as the criterion of developmental progress. All of the experiments to be reported were carried out on embryos with the extra-embryonic jelly layers intact. Preliminary runs indicated that denuding the egg served only to lower the effective concentration of the analogues. This advantage was sacrificed in favor of the utilization of larger numbers of larvae.

In cases where extreme insolubility precluded the use of a compound in the desired concentration, attempts were made to convert them into salts. The final pH of all solutions ranged between 5.5 and 7.0. When adjustment to meet this requirement had to be made, it was done by the addition of small amounts of dilute acid or base. All solutions were changed every third day.

II. After the determination of the relative effectiveness of the analogues, immersion studies were initiated at different stages of development. This was done in the hope of revealing changes in the metabolite requirements of the embryo as development progressed.

III. In each of the preceding experimental groups embryos were removed after varying periods of exposure to the antimetabolites. These embryos were rinsed in three washes of spring water and then allowed to continue to develop in spring water.

IV. Reversal experiments were carried out by the study of development in the presence of various inhibitor to metabolite ratios throughout the developmental period under consideration.

V. Sample embryos were taken for histological examination wherever significant results were obtained. These embryos were prepared by the method described by Drury ('41). All animals were oriented under a binocular dissecting microscope

before imbedding was completed. Serial sections were cut at 10 μ and stained in Galigher's modification of Harris' haematoxylin ('34) and eosin. The eosin solution was acidulated by adding 4 cm^3 of 0.1 *N* HCl per 100 cm^3 of the dye solution. Staining was accomplished by the progressive staining technique outlined by Galigher ('34).

RESULTS

The results presented in this report will be primarily concerned with the inhibition patterns as revealed by over 100 analogues tested to date. Some concomitant morphological effects and the results of attempts at reversing inhibition will also be presented. A comparative analysis of the activity of the analogues tested and of specific reversals will be presented in later papers.

In the course of presentation constant reference will be made to development in terms of Shumway stage numbers. The most noteworthy of these are stages 8–9, the period morphologically referred to as blastula; stages 11–12, the period during which gastrulation occurs; stages 14–15, the period in which the process of neurulation takes place and stages 20–23, the broad period encompassing the time from hatching until the opercular fold is formed. The terms critical periods and critical stages are used to refer to these 4 groups of developmental stages. As the succeeding results will demonstrate, they are the times at which the most significant effects were consistently noted.

In order to compare the experimental development with that of the controls, the following graphical method was utilized. The ordinate and the abscissa of the graph represent time in hours. At proper intervals, in conjunction with the time scale of the ordinate, the developmental rate of the control group is indicated by stage numbers. The experimental groups are then plotted against the controls by indicating the number of hours required to reach a comparable stage of development. Thus any group having the same development rate as the controls would graphically present a

straight line curve starting at zero hours and having a slope of unity. A decrease in rate (inhibition) would be reflected in a deflection of the curve to the right while stimulation would be visualized by a deflection to the left of the crucial

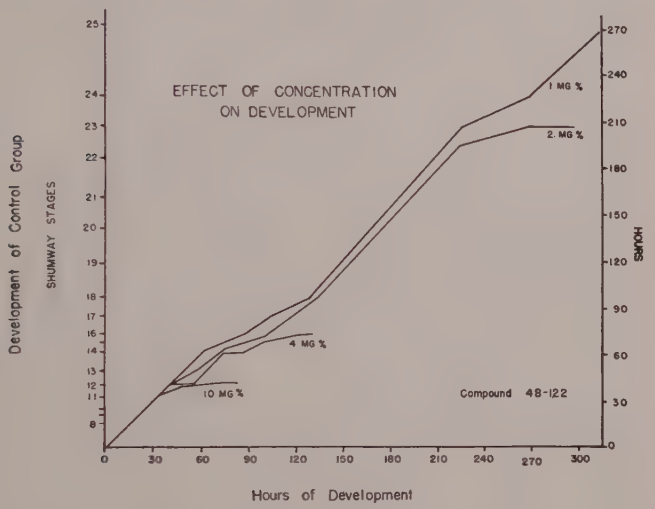

Fig. 1 Effects of various concentrations of compound 48–122 (2,4-diamino-5-p-chlorophenoxypyrimidine) on embryos immersed at the two-cell stage.

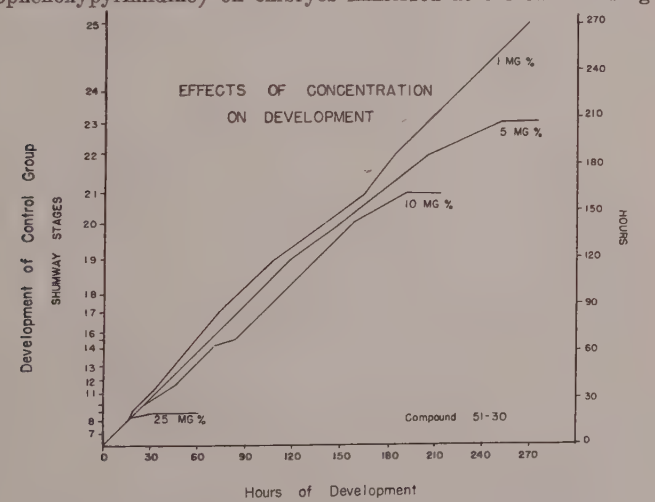

Fig. 2 Effects of various concentrations of compound 51–30 (6-mercaptopurine) on embryos immersed at the two-cell stage. Note the acceleration of development between stages 8–20 in the curve for 1 mg %.

45° line. This type of graph allows for a more critical analysis of experimental runs in which temperature fluctuations or the inherent genetic potentialities of different clotches of eggs might affect the over-all rate of development of both control and experimental animals. This is especially important in studying the earlier stages of development which encompass relatively few hours.

A. Inhibition patterns

After a preliminary screening of the analogues, several submaximal doses were chosen for each active compound and development subjected to analysis as described above. Such studies revealed that marked inhibition of the developmental rate of *Rana pipiens* can be produced at blastula (stages 8–9), gastrula (stages 11–12), neurula (stages 14–15) and the period from hatching to the formation of the opercular fold (stages 20–23). Sample developmental curves are presented in figures 1 and 2 for 2,4-diamino-5-p-chlorophenoxypyrimidine (compound 48–122) and 6-mercaptopurine (compound 51–30) respectively. Examination of the graphs reveals that if the embryo succeeded in passing through one of the critical stages it proceeded to develop at a rate comparable to that of the control group. Development continued until the next critical period when the process was repeated. It should be noted that, for these compounds, as the concentration was decreased the inhibitory effects on the earlier periods of development were diminished. If the concentration was reduced sufficiently, a slight stimulation of development was consistently noted (fig. 2). Eventually the stimulation was overcome, and in some instances was followed by a period of inhibition. The most active analogues produced inhibition at all of the critical periods; compounds of lower activity inhibited only at later stages of development. In the over-all picture the most susceptible time in development, with respect to the number of active compounds, was that period just following hatching (stages 20–23). Neurulation was next in

COMPOUND	NAME	CONCENTRATION	STAGE TREATMENT INITIATED			
			4	10	12	15
			STAGE INHIBITION PRODUCED			
		mg. %				
48–122	2,4-diamino-5-p-chlorophenoxypyrimidine	10	12	12	15	20
50–63	2,4-diamino-5 p-chlorophenyl-6-ethylpyrimidine	5	13	15	15	20
49–248	2,4-diamino-5-p-chlorophenylpyrimidine	5	12	12	14	20
49–224	2,4-diamino-5-p-chlorobenzyl-6-methylpyrimidine	20	17	17	17	22
51–30	6-mercaptopurine	20	8	12	14	21
Aminopterin		20	13	14	15	20

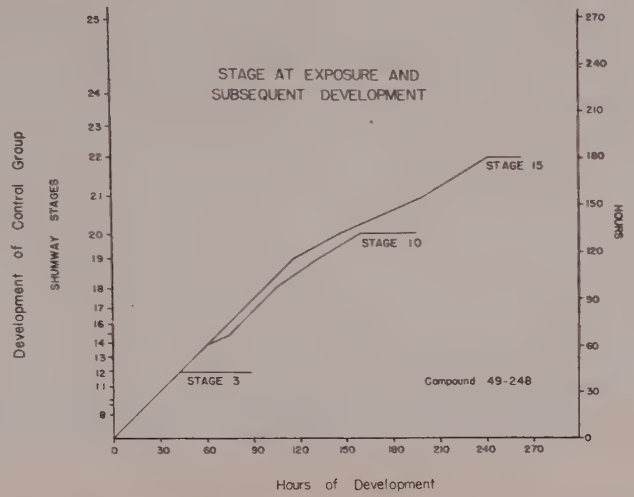

Fig. 3 Effects of initiating treatment with compound 49–248 (2,4-diamino-5-p-chlorophenylpyrimidine) at different stages of development.

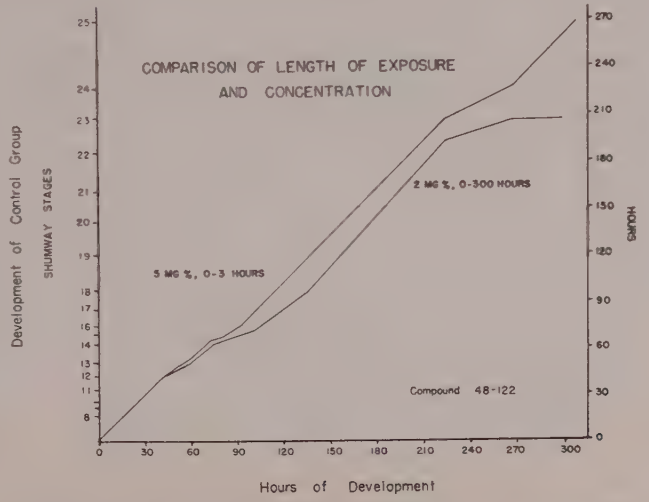

Fig. 4 Comparison of length of exposure and concentration of compound 48–122 (2,4-diamino-5-p-chlorophenoxypyrimidine) on the development of embryos immersed at the two-cell stage.

susceptibility with gastrulation and blastulation being least affected.

In most instances where treatment was initiated at different times following fertilization, development proceeded normally until the next critical period (table 1). From this stage on typical inhibition curves were obtained (fig. 3). Here, as in the earlier experiments, the degree of inhibition was related to the concentration of the antimetabolites.

The experiments in which length of exposure was varied may be summarized by stating that short exposures to high concentrations give results similar to those of long exposure at a low concentration of the same compound (fig. 4). Similar results were obtained when the analogues were applied at later stages of development. In all instances short exposures to sufficiently low concentrations resulted in a stimulation of the developmental rate.

B. Reversal of inhibition

In the course of the study of a wide variety of metabolites, e.g. folic acid, citrovorum factor, purines, pyrimidines, amino acids, and their possible role in development as evidenced by their effects on the inhibition pattern, two types of reversals were noted. The first and most obvious type of reversal was development of the metabolite-supplied group to any stage beyond that of the inhibition group of embryos. The second type of reversal, revealed in the graphs of developmental rates, was a decrease in the degree of retardation at any of the critical periods. Thus a metabolite may affect reversal by allowing development to proceed to a later stage with, however, the same apparent degree of retardation at the critical periods. Conversely, reversal may be manifested by a decrease in the degree of retardation at the critical periods while the stage at which complete cessation of development occurs is the same as the inhibition controls. Many reversals involved both types of phenomena. Examples of each type of reversal are presented in figure 5.

C. Morphological effects

The gross manifestations of successful inhibition were more or less the same for most compounds. No gross aberrations could be noted if inhibition was complete at blastula or gastrula. Cessation of development at neurula was accompanied by a persistent yolk plug in 40% of the embryos. Development beyond neurulation resulted in numerous abnormalities, the severity of which varied in proportion to the concentration

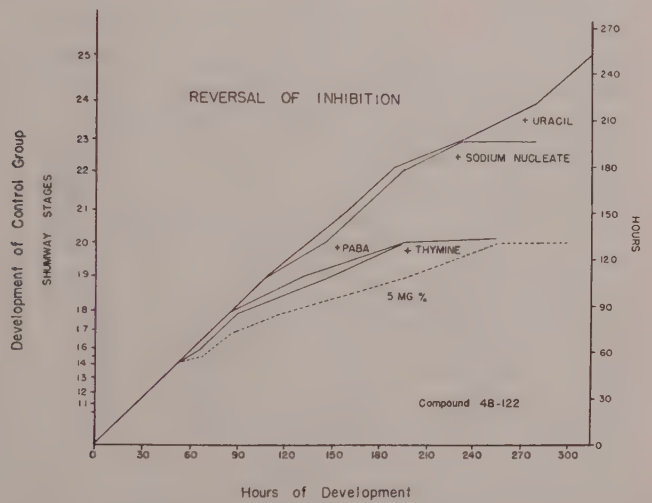

Fig. 5 Effects of the addition of various nucleic acid derivatives on the inhibition produced by 5 mg % compound 48–122 (2,4-diamino-5-p-chlorophenoxypyrimidine). The embryos were immersed at the two-cell stage.

of the inhibitor. Most prominent was a reduction in the head structures of the developing larvae, evident in some embryos as early as stage 16. Other abnormalities of the nervous system involved failure of the neural tube to fuse completely, and the appearance of cystic areas. Along with drastic neural tube involvements, poor myotomic differentiation was produced which was evidenced in the living embryo by convulsive muscular responses.

Compounds affecting early inhibition demonstrated marked ability to interfere with eye formation. Other grossly evident

abnormalities were cystic involvement of the oral suckers, aberrant gill formation, abortive and incomplete opercular development and interference with the normal formation of the intestine. Many embryos exhibited excessive proliferation of the ectoderm giving rise to characteristic epitheliomas.

Experimental combinations of the three variables, concentration, length of exposure and time of exposure, produced many histological alterations in an organ system varying from complete inhibition of differentiation to the inhibition of a specific portion of the system. Examples of these extremes of activity are presented in figures 6 to 8. Figure 6 is a section through the level of the eyes of a normal tadpole at 240 hours of development. Figures 7 and 8 represent treatment with 5 mg % and 2 mg % of compound 48–122 (2,4-diamino-5-p-chlorophenoxypyrimidine) respectively. In the first instance there was a complete inhibition of all differentiation; in the second the inhibitory activity appeared to be localized in the brain. The endodermal and mesodermal derivatives are normal. Except for the diencephalic portion, the brain, though reduced somewhat in size, revealed normal histologic development. This region was markedly inhibited with the result that the eyes developed medially so that they were almost parallel to the dorso-ventral axis of the body. Intermediate stages between these extremes were also obtained. In general the degrees of inhibition involve the following: a decrease in organ size, loss of cellular differentiation, loss of tissue organization, and finally the complete obliteration of the structure. That such severe effects may be prevented is demonstrated in figures 9 and 10 which show the effects of aminopterin and aminopterin plus citrovorum factor, respectively.

In addition to compound 48–122, compound 48–257 (2,4-diamino-5-p-chlorobenzylpyrimidine) also interfered with the normal development of the brain. Small epithelial papillae were induced in place of a lens vesicle when abnormal optic vesicle material came into contact with the overlying ectoderm (fig. 11). This interesting morphological phenome-

non indicates the possible effects these antimetabolites may have on "organizing systems."

In general, ectodermal derivatives are most severely affected while aberrations in endodermal and mesodermal derivatives are relatively less drastic. Grossly observed inhibitions and reversals are always verified by histological examination. While all the analogues affected all of the systems to some degree, there does appear to be some variation in the principal site of activity by these compounds.

DISCUSSION

Four critical periods in the early development of *Rana pipiens* may be recognized by their sensitivity to the inhibitory effects of analogues of the natural purines and pyrimidines. These critical periods precede important developmental advances. Blastula represents the earliest stage at which any synthesis has been detected, while gastrula marks the determination of the primary organizer and the genesis of the three germ layers. Neurulation precedes a marked augmentation of protoplasmic mass and the important differentiation of the axial organs. The last period, starting at hatching and ending at operculum formation, is the forerunner of a number of significant events. The most notable of these are the change from external to internal gills, the major differentiation of the various organ systems, and the final utilization of yolk before feeding begins.

The question as to whether the critical periods exist or are the result of some earlier damage to the egg by the analogue appears to be satisfactorily answered by the experiments in which the time of treatment was varied (table 1, fig. 3). In most instances inhibition became evident at the next critical period. One cannot, however, rule out the apparent accumulative effect that early treatment has on the degree of retardation at later stages of development. In the instance cited in figure 4, immersion for three hours following fertilization was sufficient to produce retardation of development 200

hours later. Either the analogue was absorbed and stored unchanged until that time when it became active, or it was incorporated into some metabolic fragment whose abnormality did not become critical until that period of development. Either interpretation implies a rather long range biochemical mobilization or, in more popular embryological terms, a rather early biochemical determination of essential metabolic intermediates.

The results presented here suggest that the 4 sensitive periods in development may be periods during which the embryo undergoes biochemical mobilization of essential metabolites. That such a biochemical mobilization might occur in advance of actual utilization is suggested by the fact established that if the embryo succeeded in passing through a critical period it was then able to develop at a rate comparable to the controls. Support for such a postulate may be found in several studies on amphibia. Breaks in the curves for oxygen consumption and glycolysis have been reported for gastrulation and neurulation (Atlas, '38; Barnes, '39; Parnas and Krasinska, '21; Trifanova et al., '39), and also in the alkaline phosphatase activity curve at late tail bud (Krugelis, '51). Plimmer and Kaya ('07) reported a decrease in phosphoprotein in the frog egg "shortly after being laid," followed by a gradual increase during development. Brachet ('47) found three distinct phases in ribonucleoprotein synthesis: an increase of only 7% between fertilization and mid-gastrula, followed by a more rapid increase to 23% by mid-neurula and 62% at hatching. Konapacki and Konapacka ('26) observed the marked utilization of yolk following neurulation. Direct chemical analysis of purine nitrogen by Graff and Barth ('38) revealed that purine synthesis started during the blastula stages and increased 100% by the feeding stage. The results fail to reveal any discontinuities in the purine synthesis curve. This would not be expected because after the first 25 hours of development the determinations were made at relatively long intervals. More recently Steinert ('52) reported the disappearance of free hypoxanthine and

guanine at early gastrula and appreciable synthesis of ATP at tailbud.

The importance of the initial metabolite supply of the egg or the condition of the ovulating female cannot be overlooked in interpreting inhibition results. It was observed that eggs obtained from females stored and starved in a cold room before maturation was complete were not only subject to inhibition by lower concentrations of the analogues but were also inhibited at stages 8–9 by groups of purine antagonists which were, in other experiments, effective only at much later stages.[3] This finding is of especial significance in view of the previously discussed data of Barth and Graff on purine synthesis and the observations of Steinert ('52) that oocytes with a maximum diameter of 0.5 mm contained no free oxypurines while mature ova contained an appreciable amount. Thus, the nutritional status of the ovulating female, her age, the season or inherent genetic makeup may account for some of the variations encountered. The results presented in this report are based on observations on over 200,000 embryos involved in repeated trials of the same compounds on many different clotches of eggs.

The morphologic response of the embryos was similar to those produced by 2,4-dinitrophenol (Dawson, '38) and chloretone (Moog, '44). Both of these compounds block cell division at late cleavage by interfering with cellular respiration. Similarly, in these laboratories 2,6-dinitrothymol was found to inhibit at stage 8, the point at which aerobic metabolism is supposed to commence. Thus any interference with the normal developmental pattern might be expected to produce similar types of aberrations. This is especially true of any compounds interfering with normal gastrular movements. A survey of the literature and a discussion of many abnormalities may be found in a recent review by Witschi ('52). The importance is not in the abnormality itself but in discovering the factors involved in its production. The successful reversal of inhibition is a step in this direction.

[3] Data to be published.

One unique morphological observation should again be noted. As seen in figure 11, in instances where amorphous optic vesicle material came in contact with the overlying ectoderm, epithelial papillae were induced in place of a lens vesicle. The implications of the phenomenon are broad. The necessity for postulating a chemical intermediate for the processes of induction and evocation has been recognized by embryologists for many years. Various investigators have relegated this key position to glycogen, protein, nucleic acids, sterols, and various combinations of these (Needham, '42). If the assumption is made that the analogues used in these experiments in some way alter nucleic acids or their synthesis, then the following inference can be drawn: the induction of epithelial papillae in place of a lens vesicle is related to the nucleic acid content of the organizer. Two possibilities exist: either an insufficient amount of nucleic acids is present or the nucleic acids present are abnormal in some way. In either case, future experiments might establish this intimate link between alterations in nucleic acid and abnormal growths.

The method herein described thus allows for the pursuit of several interesting biochemical problems. It can be used on a large scale to test compounds for inhibition of growth and thus serve as a screen for possible antitumor agents. It has the possibility of unveiling many of the fundamentals of growth and differentiation. It may also provide some clue as to the relationship between abnormal growth and abnormal metabolism.

SUMMARY

1. A method is described by which over 100 purine and pyrimidine analogues were tested for activity on developing *Rana pipiens* larvae.

2. Analysis of the developmental rates of the experimental groups as compared to the control groups revealed 4 periods in the development of *Rana pipiens* at which retardation is consistently noted: blastula (stage 8), gastrula (stages 11–12), neurula (stages 14–15) and the periods from hatching to

opercular fold formation (stages 20–23). If the embryo succeeds in passing through a critical period it then continues to develop at a rate comparable to the controls until the next critical period is attained.

3. Two types of reversal of inhibition are noted: the support of development to any stage beyond that of the inhibition group; or a decrease in the degree of retardation at the critical periods with the end point of development being the same as that of the inhibition controls. For the most part, experimental reversals consisted of a combination of both types.

4. Some gross morphological and histological observations are presented with the inhibition and reversal data.

5. The possible significance of the data is discussed and several interpretations offered.

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I would also like to thank Dr. Seymour Hutner and Dr. Eli Goldsmith for their encouragement and constructive criticism.

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PLATE 1

EXPLANATION OF FIGURES

6 Section through the level of the eyes of a normal tadpole of 240 hours. Note position of the eyes and extent of differentiation of the brain. × 33.

7 Section through the head of a tadpole treated for 240 hours with 5 mg % compound 48–122 (2,4-diamino-5-p-chlorophenoxypyrimidine). Note the complete absence of a brain and the lack of endodermal and mesodermal differentiation. × 64.

8 Section through the level of the eyes of a tadpole treated for 240 hours with 2 mg % of compound 48–122 (2,4-diamino-5-p-chlorophenoxypyrimidine). Note the reduction in brain size, particularly the diencephalon, resulting in the eyes being pulled medially and ventrally. × 38.

9 Section through the level of the hindbrain of an animal treated for 240 hours with 5 mg % of aminopterin. Note the complete absence of a brain and the presence of a small heart. × 38.

6

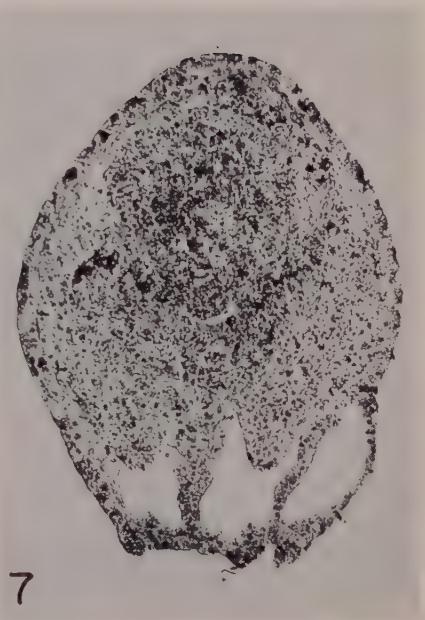

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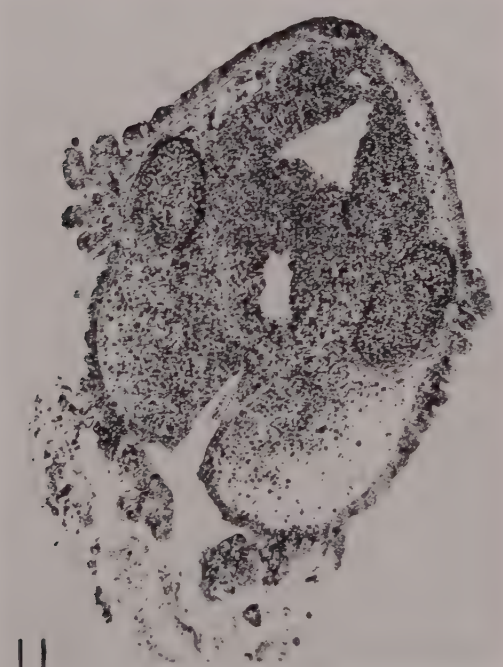
PLATE 2

EXPLANATION OF FIGURES

10 Section through the level of the hindbrain of an animal treated for 240 hours with 5 mg % aminopterin in the presence of 200 mg % citrovorum factor (Leucovorin, Lederle). Note the completely normal differentiation of all structures. × 41.

11 Section through the level of the eyes of a tadpole treated for 240 hours with 2 mg % of compound 48–257 (2,4-diamino-5-p-chlorobenzylpyrimidine). Note the induction of epithelial papillae in place of a lens vesicle and the lack of cellular differentiation in the brain. × 64.

10



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THE EFFECT OF X-IRRADIATION ON THE GROWTH AND HISTOCHEMISTRY OF ADRENAL AUTOTRANSPLANTS IN THE RAT [1]

LELAND C. WYMAN, RAE WHITNEY, PATRICIA L. GRIFFIN AND DONALD I. PATT [2]

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The possibility of accidental exposure to severe irradiation creates the necessity of improving grafting techniques, if possible, and in addition developing methods to encourage the prompt regeneration of injured tissues. Many workers have provided a background of basic research by exploring in various species the effect of irradiation on mortality and on the morphology and functioning of various organs and organ-systems including the adrenal gland (Bloom, '48; Cronkite and Chapman, '50; Edelmann, '51; Ellinger, '48; Graham et al., 50; Knowlton and Hempelmann, '49; North and Nims, AECU-266; and many others). Most of these experimenters used sublethal or threshold irradiation doses and the general conclusion was that radiation effects on the adrenal with the doses reported are not peculiar in their nature but are similar to those of other non-specific stresses.

Of those who have investigated the effects of irradiation on the morphology of tissues and individual cells, Fogg and Cowning ('51) and Halberstaedter and Ickowicz ('47) found that when damage occurred in cells the degree of injury was in proportion to the magnitude of the dose; Knowlton and Hem-

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pelmann ('49) found no correlation between radiosensitivity and interference with mitosis in the mouse; Warren et al. ('50) found that the adrenal cells seem to show no specific effect following beta irradiation. Most of those who have noted the effects of irradiation upon mitosis in particular find that low doses of x-irradiation depress cell division, but that recovery occurs subsequently (Evans, '50; Hevesy, '52; Knowlton et al., '48; Reid and Gifford, '52). La Cour ('51), however, records an increase in mitosis in irradiated *Trillium* root tips. Glucksmann ('52) concluded that the cure of some tumors may depend upon the depression of cell division accompanied by continued ability to differentiate. Toosy ('51) found that epidermis treated with methylcholanthrene showed only minor differences from irradiated epidermis, indicating that the radiation effect was not specific.

A direct study of the effect of irradiation on tissue grafts was made by Ungar and Warren ('37) in the rabbit, in which they found that corium, severely irradiated with gamma and beta rays, could maintain neither itself nor epidermis grafted to it. Irradiated epidermis, on the other hand, was capable of growing on non-irradiated corium in 32% of the cases. From their results they concluded that death of the epidermal graft was due to a combination of indirect and direct effects of radiation.

Since severe overdosage with irradiation has prevented the successful grafting of skin, and since the adrenal structure is not readily damaged by irradiation an attempt was made to test further the effects of irradiation upon grafted tissues by the use of adrenal autotransplants in rats. Although the reports cited above generally indicate that the effects of irradiation are non-specific, it was thought that there might be some demonstrable difference between the success of regeneration which follows direct irradiation of the gland to be transplanted, and that which follows irradiation of the site of implantation. If so, the findings might have some bearing on surgical procedure when tissue grafts are necessary.

MATERIALS AND METHODS

Male rats of the Wistar strain weighing from 80 to 120 gm at the time of autotransplantation were used. Purina laboratory chow and tap water were given *ad libitum*. Using sodium pentobarbitol [3] anaesthesia, transplants were made according to the method of Wyman and tum Suden ('37) and were recovered (when present) 11 to 14 days following the operation. In order to demonstrate ascorbic acid the transplants were fixed in acidified silver nitrate and alcohol (Gomori, '52), sectioned in paraffin at 5 μ and stained with haematoxylin and eosin. To visualize lipids, neutral formalin fixation was used, followed by sectioning of frozen tissues at 15 μ and staining with Sudan IV or Oil red O. Unstained frozen sections were used for the study of birefringence with a polarizing microscope.

For determination of the amount of regeneration (cell depth) and for analyzing the histochemical characteristics of any given transplant, the cross-section was used which contained the deepest cell layer. Cell depth was determined by counting the number of nuclei lying between the peripheral and central limits of the regenerating mass as it appeared in cross-section. Per cent mitosis was determined by counting nuclei and mitoses in 10 different areas in each of the cortical zones, using an ocular grid under 430 × magnification. Counts were done on 10 transplants from each experimental group except one (5 in this group). Statistical analysis included the use of the Snedecor *F*-test, the Fisher *t*-test and an analysis of difference between proportions. The 5% level of significance was employed throughout.

Two series of experiments will be reported, and since the methods used for them differed somewhat, they will be discussed separately.

Irradiation of adrenal glands in vitro (gland-irradiation). Glands were placed in mammalian Ringer's soluton immediately upon removal and x-irradiated in this solution at 100 r per minute for 20 minutes (total dose 2000 r). The maximum

[3] Nembutal, Abbott.

capacity of the machine was 150 KVP, 8.5 ma.; inherent filtration equivalent to 0.5 mm aluminum. For this study, the machine was run generally at 145 KVP, 6.5 ma., focal distance of 10 inches, no filters. After irradiation each gland was bisected and the 4 pieces transplanted to the muscles of the back. Controls were of two kinds; those implanted after isolation *in vitro* (Ringer's) for a period (about 50 minutes) equivalent to that necessary for the *in vitro* irradiation procedures (isolated controls); and those which were implanted immediately upon removal from their normal site without an intervening period of isolation (untreated controls).

Local irradiation of the transplantation site (site-irradiation). The rats were adrenalectomized, the right adrenal was discarded and the other was isolated in mammalian Ringer's until time for its implantation. Meanwhile the animal was placed in a lead box with openings arranged to allow local irradiation of a small spot of exposed musculature, 6 mm in diameter, on each flank. This site was chosen instead of the usual one in the muscles of the back to avoid possible effects of irradiating underlying viscera. Total x-ray dosage of 2000 r (100 r per minute) was given. The irradiated muscles were marked with a red pencil [4] for identification. The left adrenal was removed from Ringer's, bisected, and the two pieces implanted in the locally irradiated, marked areas. Controls were of two sorts; those isolated in Ringer's for a period of time equalling that necessary for irradiation of the site and then implanted into sites marked with red pencil (marked controls); and those isolated and implanted into non-irradiated, unmarked sites (unmarked controls).

RESULTS AND DISCUSSION

Per cent successful take. Irradiation of the glands with 2000 r before implantation reduced (to 67%) the ability of the implants to become established in their new sites and irradiation of the sites reduced the establishment of implants to 75% (see table 1). In neither the gland-irradiation nor the site-

[4] Blaisdell — Ben Franklin Indelible no. 561-Red.

irradiation series was there a significant difference between the two control groups.

Cell depth. When regeneration had occurred a marked difference was seen by 14 days between the gland-irradiated and site-irradiated transplants. In the former (fig. 3) cell-depth was about a third that of the controls (figs. 1, 2) while in the latter there was no difference between the experimental and control transplants. The various types of controls did not

TABLE 1

Effect of x-irradiation on regeneration and histochemistry of 14-day adrenocortical autotransplants in rats

	REGENERATION			HISTOCHEMISTRY		
	Per cent successful take	Average cell depth	Per cent mitosis	Number without ascorbic acid	Number without sudanophilic lipid	Number without birefringence
	Gland-irradiation					
Untreated controls	92 (36)[1]	42 (33)	0.86 (10)	2 (12)	0 (12)	4 (7)
Isolated controls	86 (15)	34 (13)	**1.17** (10)	2 (13)	0 (11)	**3** (7)
Irradiated 2000 r *in vitro*	67 (18)[4]	**12**[2] (12)	**0.63** (10)	**7** (10)	**1** (10)	**3**[3] (7)
	Site-irradiation					
Unmarked controls	100 (7)	36 (7)	0.70 (5)	2 (7)	0 (5)	1 (5)
Marked controls	100 (10)	33 (10)	**0.41** (10)	1 (10)	3 (9)	**9** (9)
Irradiated 2000 r	**74** (23)	33 (17)	**2.03** (10)	**10** (17)	3 (16)	**13**[3] (16)

[1] Numbers in parentheses signify number of rats in the group.

[2] Values in boldface are significantly different from values of control group just preceding.

[3] Significant difference exists between site-irradiated and gland-irradiated transplants.

[4] A significant difference exists between this value and that of the untreated controls.

differ significantly from one another. Apparently a 50-minute isolation or marking the site did not affect their growth capacity. Since x-irradiation is known to suppress mitosis and cell division (Hevesy, '52) and to destroy cells (Fogg and Cowning, '51; Reid and Gifford, '52), it is reasonable to suppose that the successful, gland-irradiated transplants, even though established and vascularized, were seriously handicapped in their early days of growth by inhibition of cell division which caused them to fall behind all other types of transplants. Glands not directly irradiated, however, even though placed in irradiated sites, if successful, were able to equal the cell production of the isolated controls. An irradiated environment, therefore, does not seriously affect the growth capacity of an adrenal implant once it becomes established although it does reduce the chances of a successful take.

Per cent mitosis. The supposition that mitosis in irradiated glands was suppressed is supported by the low mitotic index found in the gland-irradiated transplants (table 1). In view of the small amount of regeneration in these transplants it is apparent that this depression of mitosis had persisted since implantation of the gland. Control implants, however, regardless of their mitotic index at 14 days, showed a considerable amount of regeneration, indicating that the treatment they had undergone (isolation, marking) had not impaired their growth capacity. Although the implants placed in irradiated sites but untouched themselves by x-ray had a cell depth no greater than that of the unmarked controls, their mitotic index was the highest of any group. This may mean that their mitotic activity was never severely depressed (Knowlton and Hempelmann, '49) and that at 14 days their index represented a delayed peak of "overcompensation" (Canti and Spear, '29). Curves showing over-shooting of the normal values were reported by Knowlton et al. ('48) in a study of the effects of x-rays on the mitotic activity of mouse epidermis in which a dose of 325 r caused an immediate suppression of mitosis on day zero followed by slow recovery through day 4, a high peak of mitotic activity at 8 days following irradiation, and a return to normal

at about 12 days. Although this effect followed direct irradiation of a tissue, indirect effects are also known (Hevesy, '52). Summarizing, it appears that there is a prolonged depression of cell division in tissues attempting to regenerate following direct irradiation with 2000 r. The indirect effects of site irradiation, on the other hand, are transitory and at 14 days the tissues show signs of vigorous regenerative activity.

Ascorbic acid. Direct irradiation of the gland in most cases interfered with the ability to exhibit ascorbic acid as indicated by a complete absence of silver granules in 7 out of 10 transplants (table 1). Irradiation of the implantation site had a similar effect. It should be stated here that we have no way of knowing at present whether the transplants lacking ascorbic acid at 14 days would not exhibit it later if allowed to continue their growth. Lack of ascorbic acid need not imply that the tissue is incapable of secretory activity. It could indicate a high secretory rate in which materials are utilized as rapidly as they become available.

In order to evaluate the tissues in which ascorbic acid appeared, an arbitrary scale was devised. It is believed that fine granulation of silver (ascorbic acid), lipids or birefringent particles denotes a state of secretory activity in the gland (Deane et al., '48; Deane and Morse, '48; Greep and Deane, '49; Weaver and Nelson, '43); therefore a value of 7 was assigned to the smallest granules (fig. 11), and at the opposite end of the scale, a value of one for large, coarse granules, denoting storage (fig. 8). The numbers of granules relative to the intact normal gland were indicated by a scale from one through 5, in which 4 represented the maximum normal condition. The two values for size and number for each of the zones of the transplant cross-section were multiplied together. The sum of these products served as an index of secretory capacity for the transplant and their average served as an index of the secretory activity of its individual cells. Although the evaluation was highly subjective, every attempt was made to set rigid criteria and to judge size and number of granules as consistently as the human variable will allow. For any given trans-

plant, the cross-section chosen for this analysis was that having the greatest cell depth.

Irradiation of the gland before implantation reduced the index of capacity by 71% whereas irradiation of the site only reduced it by 21% (table 2 and fig. 12). The secretory capacity of a transplant would depend upon its size and since there was considerable variation in cell depth there was likewise a varia-

TABLE 2

Effect of x-irradiation on histochemistry of 14-day regenerating adrenocortical autotransplants in rats

	ASCORBIC ACID (activity index)[1]	ASCORBIC ACID (capacity index)[2]	SUDANOPHILIC LIPIDS[2]	BIREFRINGENCE[2]
	Gland-irradiation			
Untreated controls	22 (10)[3]	63 (10)	44 (12)	7 (3)
Isolated controls	20 (11)	51 (11)	49 (11)	33 (4)
Irradiated 2000 r *in vitro*	16 (3)	18 (3)	**21**[4] (9)	16 (3)
	Site-irradiation			
Unmarked controls	15 (5)	51 (5)	69 (5)	32 (4)
Marked controls	8 (9)	32 (9)	33 (6)	.. (0)[5]
Irradiated 2000 r	10 (7)	40 (7)	28 (13)	15 (3)

[1] $\frac{\frac{\Sigma \text{ Number of granules} \times \text{size of granules}}{N}}{\text{Number of zones}}$.

[2] $\frac{\Sigma \text{ Number of granules} \times \text{size of granules}}{N}$.

[3] Numbers in parentheses signify number of rats in the group.

[4] Values in boldface type differ significantly from values of control group just preceding.

[5] None of these transplants exhibited birefringence.

tion in the number of zones represented among the groups of experimental and control transplants. The differences, therefore, between the experimental (figs. 11, 12) and the control (figs. 9, 10) groups would depend to a large extent upon the amount of regeneration which had taken place and would not necessarily reflect the true secretory activity of the individual cells. Although the gland-irradiated transplants had the lowest index of activity, as might be expected, such differences between the groups in both the gland-irradiated and the site-irradiated series were very slight (table 2). Apparently, when the tissue is capable of exhibiting ascorbic acid, the cells do so at about the same level of activity, regardless of their treatment. Studies are now in progress attempting to correlate the morphological and histochemical findings with the functional efficiency of irradiated and non-irradiated implants through the application of various stress tests.

Sudanophilia and birefringence. The data concerning lipids appear equivocal (tables 1 and 2) and do not lead to safe conclusions regarding irradiation effects. Nevertheless they are reported here for those who may be interested in their behavior. All 14 day transplants (figs. 4, 7), whether control or experimental, were both sudanophilic- (fig. 6) and birefringence-poor compared with an intact, normal gland (fig. 5); only close observation showed that lipids were not entirely absent. In such a condition it might be easy to induce complete removal of the small amount present by injudicious experimental procedures, and studies involving these characteristics might better be made when the transplants are more mature and the lipid metabolism is less delicately balanced.

More transplants lacking sudanophilic and birefringent particles appeared in the groups in which a marking dye was used (table 1). Perhaps some slight toxicity of the dye upset the delicate balance mentioned above. For the transplants in which lipids could be demonstrated (table 2), the capacity indices for sudanophilia and birefringence were calculated by the same arbitrary scale as for ascorbic acid *(v.s.)*. Following gland-irradiation (fig. 6) the capacity index for sudanophilia was

48% below that of the controls, correlating closely with the behavior of the ascorbic acid for this index. Site-irradiation transplants also showed a reduction in sudanophilia, but so did the marked controls, the implication being that marking the sites in some way interfered with the lipid metabolism of the transplants.

SUMMARY AND CONCLUSIONS

The effects of direct x-irradiation of the adrenal glands of rats or of local irradiation of the site of implantation, with 2000 r, on the subsequent growth of intramuscular, autoplastic, adrenocortical transplants was studied. Site-irradiation was more effective in preventing successful establishment of implants than was direct gland-irradiation. But once the tissue was established, its ability to grow rapidly and to assume the histochemical characteristics of normally regenerating cells was affected more by direct irradiation than by irradiation of the site of implantation. Gland-irradiation had a prolonged depressing effect on mitotic activity, while site-irradiation had only a temporary effect. Values for lipids determined by sudanophilia and birefringence were equivocal, but failure to exhibit ascorbic acid was induced both by gland- and by site-irradiation. Analysis of the data showed that when the transplant was capable of exhibiting ascorbic acid the cells did so at about the same level of activity, regardless of their treatment. Total secretory capacity, however, was reduced more by gland- than by site-irradiation, since it depends largely on the size of the transplant rather than on individual cell performance.

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PLATE 1

EXPLANATION OF FIGURES

Photomicrographs of regenerating adrenocortical tissue at 14 days after autotransplantation. H. and E. stain unless otherwise indicated.

1 *Untreated control.* Actively dividing cells with crowded nuclei and little cytoplasm. Arrow indicates inner limit of regenerating tissue. Cell depth 50 (average for this group, 46 cells). × 100.

2 *Isolated control.* Cell depth 35 (average for this group, 36 cells). × 100.

3 *Gland-irradiated transplant.* 2000 r *in vitro.* Adrenocortical cells limited to a small area (see arrows). Cell depth 11 (average for this group, 12). × 100.

4 *Untreated control.* Stained with Oil red O. The light outer band is the new regenerating tissue (compare with fig. 7). The inner area of old degenerating tissue still retains much of its original lipid which has been phagocytized by macrophages (see arrow). × 100.

5 *Intact adrenal gland.* Oil red O. Numerous sudanophilic particles. × 930.

6 *Gland-irradiated transplant.* Oil red O. Newly regenerated cells at top are lipid-poor. Original implant cells below, retain abundant sudanophilic particles. × 930.

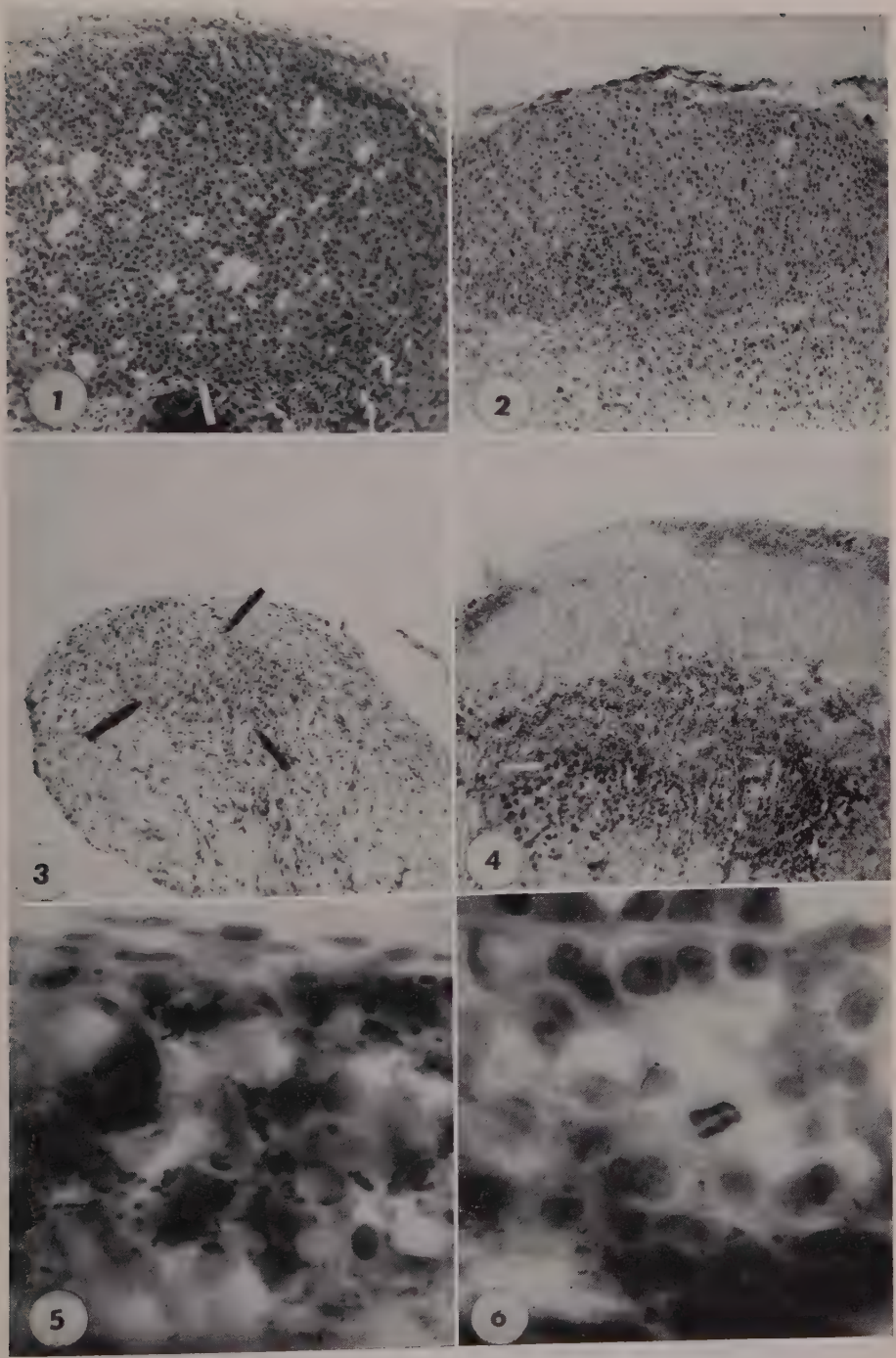
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PLATE 2

EXPLANATION OF FIGURES

7 *Gland-irradiated transplant.* 2000 r *in vitro.* The outer zone of regenerating tissue (see arrow) is almost devoid of sudanophilic lipids (compare with fig. 4). The inner area of old degenerating tissue contains abundant lipids staining like those of an intact gland. × 100.

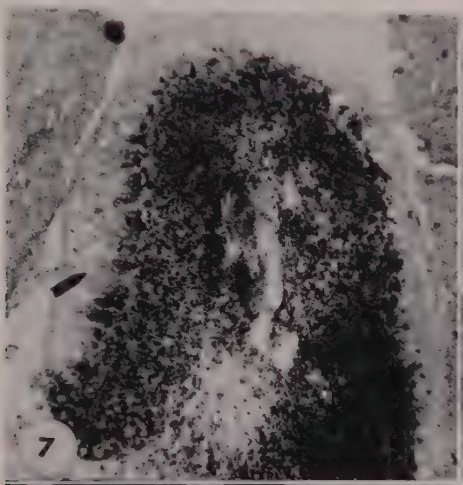
8 *Intact adrenal gland.* Silver granules indicate presence of ascorbic acid. Medium and coarse granules indicate storage rather than active secretion. × 930.

9 *Untreated control.* Silver granules numerous but slightly finer than those of intact gland. × 930.

10 *Isolated control.* Silver granules finer and less numerous than in figure 9. × 930.

11 *Gland-irradiated transplant.* 2000 r *in vitro.* Silver granules rare, and when present, are very fine (see arrow). × 930.

12 *Site-irradiated transplant.* 2000 r. Silver granules similar in size to those of intact gland and untreated control but less numerous. × 930.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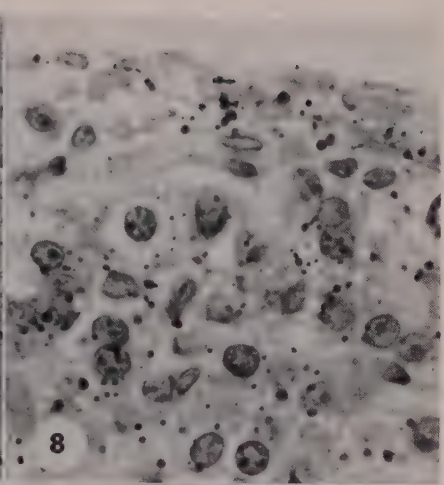
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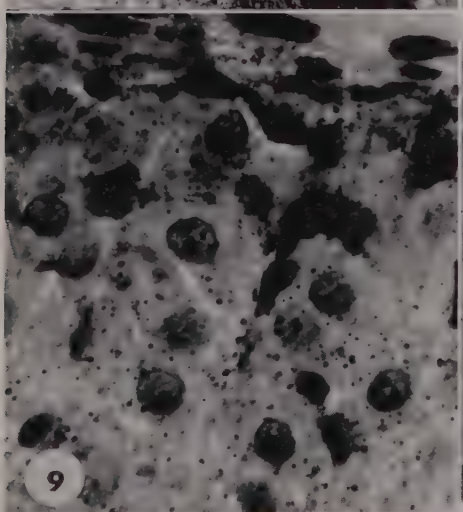
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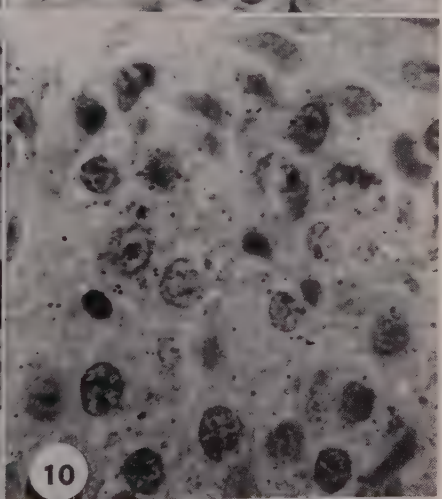
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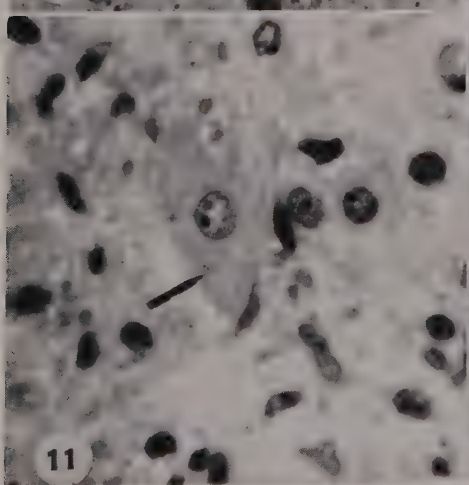
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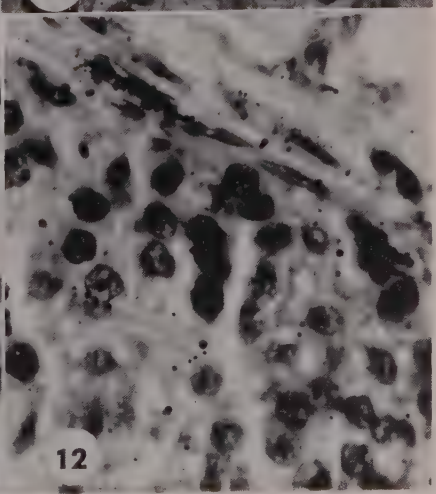
12

INACTIVATION OF CYPRIDINA LUCIFERASE BY UREA[1]

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FOUR FIGURES

INTRODUCTION

Denaturation of proteins by urea has received a great deal of attention. Some of the earlier studies are cited in the paper by Steinhardt ('38) and in the general review of protein denaturation by Neurath et al. ('44). The papers of Kauzmann et al. ('53) contain numerous references to more recent work.

In contrast with the relatively great amount of work involving the effects of urea upon proteins in general, its influence upon enzyme activity does not appear to have been very much studied, although the rate of an enzyme-catalyzed reaction would seem to be an excellent criterion. We have been able to find relatively few papers in which enzyme activity was utilized for study. Some examples are the experiments of Bhagvat et al. ('39) on amine oxidase, the work of Deutsch ('51) utilizing crystalline horse erythrocyte catalase and Dzialoszynski's ('51) investigations with phenolsulfatase.

The enzyme, luciferase, from the luminescent ostracod crustacean, *Cypridina hilgendorfii,* has been extensively studied in other connections (Harvey, '52). While not yet crystallized, it has recently been considerably purified (McElroy and Chase, '51). The blue luminescence of the luciferase-luciferin reaction, which is an instantaneous measure of its rate, can be determined with rather high precision by the

[1] This research was supported in part by funds of the Eugene Higgins Trust allocated to Princeton University.

method of Anderson ('33). His apparatus utilizes a photocell, a condenser, a potentiometer, and a Lindemann electrometer to integrate the light produced as a function of time. The equation for a first order reaction closely describes the process and the relative rate constant very satisfactorily represents the enzyme activity (Chase, '50).

Since exploratory qualitative experiments had indicated that the light intensity is affected by urea, it seemed desirable to study this quantitatively, and experiments were therefore designed to determine the relationship between activity of the enzyme and urea concentration and to ascertain whether the effect of urea might be reversible. As will presently be shown, urea apparently affects luciferase in two distinct ways but is without effect upon the substrate, luciferin.

MATERIALS AND METHODS

The luciferase was obtained by a modification of the method of McElroy and Chase ('51). Only the first two steps of their procedure were used, their Fraction II representing the final product in this case. The starting material consisted of 25 gm of dry *Cypridina* organisms, freshly ground for two hours in a ball mill. The resulting powder was extracted for 24 hours with benzene. After evaporating this off, the powder was subjected to the first half of the procedure of McElroy and Chase ('51) and Fraction II was stored in the deep freeze at — 15°C. A small amount was diluted 1 : 100 with water whenever an enzyme stock solution was needed. There was no measurable loss of activity over a period of 6 months at — 15°C. The absorption spectrum of this luciferase solution is shown in figure 1. It is clearly a simple protein spectrum, with a maximum at 278 mμ.

The luciferin used as substrate in the luminescent reaction was obtained by extraction of 20 gm of benzene-treated *Cypridina* powder with 250 ml of methanol in a hydrogen atmosphere. After 24 hours, the solid residue was removed by centrifuging and the transparent, orange-yellow supernatant

was stored under hydrogen.[2] There was no significant difference between results using this luciferin stock solution and those with luciferin that had been purified by the more laborious method of Anderson ('35).

Because pH, salt concentration and ion content all affect this luminescent reaction (Anderson, '33; Chase, '48), the luciferase, luciferin and urea solutions were always prepared

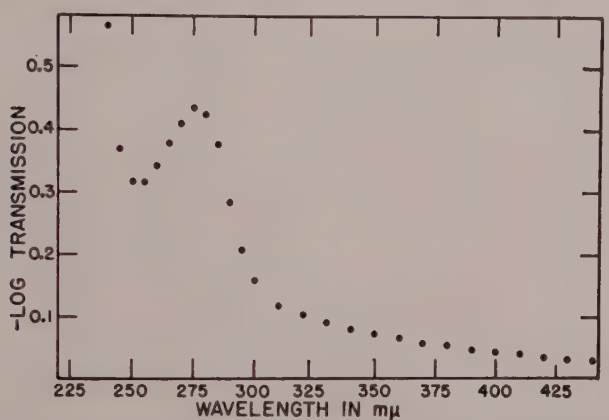

Fig. 1 The absorption spectrum of the luciferase preparation used in the present work. Although not plotted here, measurements have actually been extended as far as 750 mμ without finding any visible absorption. A simple protein absorption spectrum is indicated, with one maximum at 275 mμ — 278 mμ.

in a particular "reaction mixture" consisting of equivalent amounts of KH_2PO_4 and Na_2HPO_4 to give a 0.067 *M* phosphate concentration. Hundredth molar NaCl was also present. The pH was 6.8.

Baker's C. P. urea was used to make 5.0 *M* stock urea solutions, which were kept in the refrigerator. Such a solution was not used for longer than 24 hours.

Unless otherwise stated, the experiments were done as follows. Having determined the proper concentrations of luciferase and luciferin for the luminescent reaction to be over in about three minutes, these amounts were regularly used

[2] Dissolved *Cypridina* luciferin is very unstable in the presence of air. This instability is probably due to an oxidative process and is retarded at low temperatures and low pH and abolished if oxygen be removed from the solution.

and only the concentration of urea was varied. The standard quantity of luciferase stock solution was measured into a small beaker and 10.0 ml of reaction mixture were added. Next, the standard quantity of luciferin stock solution (kept in an ice water bath to retard oxidation[3]) was delivered into the reaction vessel of the light-measuring apparatus and 20.0 ml of reaction mixture were added, containing sufficient urea to give the desired concentration when the luciferase solution was finally run in to start the luminescent reaction. A stop-watch was simultaneously started and the total light emitted, expressed in millivolts as read from the potentiometer of the apparatus, was recorded as a function of time. Such data represent *total light emitted,* and are comparable to measuring the concentration of an end product.

Since it was the effect of various concentrations of urea upon luciferase activity that was of interest, the relative first order rate constant was determined for each experiment. This was done by plotting the Briggsian logarithm of the amount of light (i.e., proportional to millivolts as indicated by the potentiometer reading) still to be emitted against time from the beginning of the reaction, and calculating the slope of the resulting straight line. Three such determinations were ordinarily made and their values were averaged to give the enzyme activity for a particular urea concentration. Individual slopes obtained in this manner were usually very reproducible, as is evident from the values in table 1.

Figure 2 shows the data from 5 individual luminescence experiments at concentrations of urea from zero to 1.0 *M*. Log $(a - x)$ has been plotted against time and it is quite evident that the equation for a first order reaction holds and that the relative luciferase activity can be ascertained rather precisely.

It should be mentioned that the pH of the reaction mixture is practically unaffected by urea over the concentration range studied, so that possible effects of hydrogen ion concentration on the luciferase activity (Chase '48) need not be considered in these experiments.

[3] See footnote 2, page 51.

TABLE 1

Relative first order rate constants for the luminescent reaction of standard amounts of luciferase and luciferin in the presence of various concentrations of urea

UREA CONC.	EXPER. A	EXPER. B	EXPER. C	AVERAGE
0.00 *M*	0.393	0.396	0.394	0.394
0.20	0.325	....	....	0.325
0.25	0.339	0.329	0.325	0.331
0.30	0.288	....	....	0.288
0.50	0.224	0.220	0.225	0.223
0.75	0.179	0.179	0.178	0.179
1.00	0.127	0.106	0.108	0.114
1.50	0.057	0.058	0.059	0.058

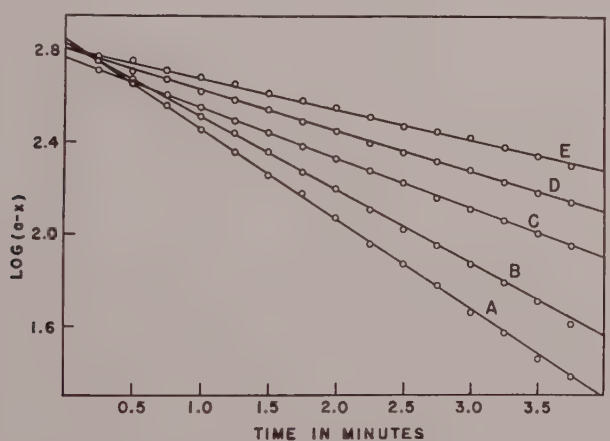

Fig. 2 Typical luminescence measurements analyzed in terms of a simple first order reaction equation, by plotting Log (a — x) against time. Since straight lines result, the reactions are clearly first order. In experiment A no urea was present. In experiments B, C, D, and E, 0.25 *M*, 0.50 *M*, 0.75 *M*, and 1.00 *M* concentrations were present, respectively. The slopes of the lines which fit the data are proportional to the luciferase activities.

EXPERIMENTAL RESULTS

Three results came out of this work. First, an immediate inactivation of luciferase exists, varying in extent with urea concentration. Second, this effect is reversible. Third, a gradual inactivation of the enzyme by urea also occurs, apparently quite distinct from the immediate effect.

Immediate inactivation of luciferase by urea. By running the luciferase-luciferin reaction in the presence of various concentrations of urea (all other conditions being maintained constant) the results shown in figure 3 were obtained. Most of the points in the figure are based upon the average of three luminescent reactions. Per cent luciferase activity is plotted against the logarithm of the urea concentration. The per cent

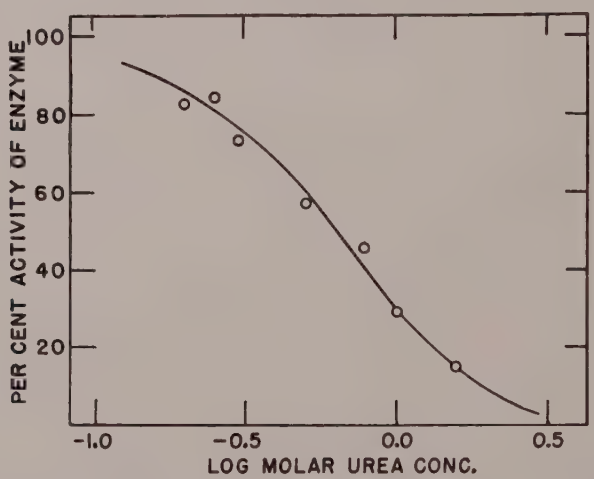

Fig. 3 Per cent activity of luciferase plotted against the logarithm of the urea concentration. Activities have been calculated from the data of table 1, as described in the text. The sigmoid curve was fitted to the points by eye. Although not a theoretical curve, it is the sort that might be expected if a complex between luciferase and urea is formed reversibly.

activity for any given concentration of urea is found by dividing the relative first order rate constant of the reaction run in presence of that urea concentration by the relative rate constant of the reaction in absence of urea. Table 1, already referred to, contains the individual and averaged values of the relative first order reaction rate constants that were used in calculating the per cent activity values used in the construction of figure 3.

A sigmoid curve has been fitted to the points in figure 3, although this is not necessarily the only curve that could be

fitted to these data. It is quite apparent that the enzyme activity is greatly affected by urea, and it would appear that the concentration of urea required to produce an inactivation of 50% (about 0.6 *M*), is considerably lower than is the case for denaturation of most proteins, where urea concentrations of the order of 6 to 8 molar seem to be ordinarily needed. As stated above, we have been able to find only a few instances where enzyme activity has been studied as a function of urea concentration. In two of those cited (i.e.; Bhagvat et al., '39, and Deutsch, '51), urea concentrations of the order of 5 and 6 molar were used. These resemble those concentrations required for ordinary protein denaturation. It must be remembered that the method of measurement of luciferase activity permits the recognition of an almost instantaneous effect of the urea on the enzyme. It is, therefore, quite possible that similar immediate effects of urea on the amine oxidase and the catalase studied by Bhagvat et al. and by Deutsch may exist but missed being observed because of the time required for measurement.

Reversibility of inactivation by urea. Choosing a urea concentration that caused about 50% inactivation of the luciferase, it was demonstrated that the effect is reversible. The method used involved dilution and was similar to that described by Chase and Brigham ('52) for demonstrating reversible inactivation of this same enzyme by butyl alcohol.

In the present case, three series of experiments were performed, each consisting of three separate luminescent reactions. In the first series luciferase and luciferin alone were present, without any urea. In the second series, the luciferase was first exposed to a 0.6 *M* concentration of urea but upon addition of the luciferin solution (lacking urea) the concentration was decreased, by dilution, to 0.3 *M*. In the third series luciferase and luciferin and a 0.6 *M* urea concentration were present throughout.

If the combination of luciferase and urea were irreversible, the luciferase activities obtained in the experiments of series two and three should be the same because dilution of the urea

concentration to one-half, in series two, should not cause dissociation of the luciferase-urea compound. If, on the other hand, the combination were easily reversible, the enzyme activities from the experiments of series two should lie midway between those of series one and series three, because dilution by half of the urea concentration to which the enzyme was originally exposed in series two should cause a corresponding dissociation of the luciferase-urea compound.

TABLE 2

Experimental demonstration of the reversible nature of the inactivation of luciferase by urea. See text for details

INITIAL UREA CONC. (molarity)	FINAL UREA CONC. (molarity)	RELATIVE RATE CONSTANTS FOR THREE INDIVIDUAL EXPERIMENTS			AVERAGE VALUE OF RATE CONSTANT	PER CENT OF ACTIVITY
0.0	0.0	0.694	0.681	0.679	0.685	100.0
0.6	0.3	0.510	0.526	0.515	0.517	75.5
0.6	0.6	0.365	0.358	0.366	0.363	53.0

Table 2 gives the results of these experiments. It is quite clear that the per cent activity obtained from the experiments of series two lies about midway between those found for series one and series three, indicating that the reaction of urea with luciferase is reversible under the present experimental conditions. Another series of experiments was also performed, which yielded a similar result.

The inactivation of luciferase by urea appears, then, to be a reversible process and it seemed of interest to analyze the data further. If it be assumed that the enzyme combine reversibly with urea to form an inactive compound, an ordinary mass law equation should apply:

$$\text{luciferase} + a\ \text{urea} \rightleftharpoons \text{luciferase (urea)}_a$$

where a is the number of molecules of urea which combine reversibly with one luciferase molecule (or catalytic center on the molecule) to cause inactivation. This can be stated as follows:

$$\frac{[\text{luciferase}]\ [\text{urea}]^a}{[\text{luciferase (urea)}_a]} = K$$

where K represents the dissociation constant of the inactive luciferase-urea complex.

Rearranging:

$$\frac{[\text{luciferase}]}{[\text{luciferase (urea)}_a]} = K \cdot [\text{urea}]^{-a}$$

and:

$$\text{Log } \frac{[\text{luciferase}]}{[\text{luciferase (urea)}_a]} = -a \text{ Log } [\text{urea}] + \text{Log } K$$

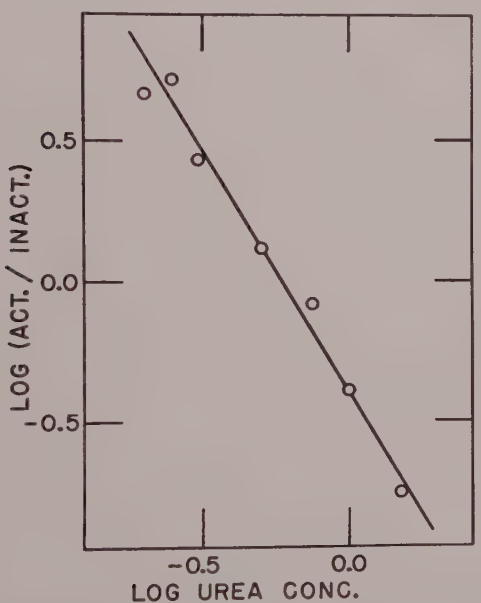

Fig. 4 The data of table 1, analyzed in terms of a simple, mass law relationship. A straight line fits the points fairly well and its slope, 1.70, should indicate the number of urea molecules which combine reversibly with each luciferase molecule, or active catalytic site, in forming the inactive urea-luciferase complex. See text for further discussion.

Since [luciferase]/[luciferase (urea)$_a$] equals the fraction of the total enzyme possessing activity divided by the fraction of the total enzyme that is inactive, a plot of the logarithm of the ratio of the active fraction to the inactive fraction against the logarithm of the urea concentration would be expected to give a straight line if this equation applies. Furthermore, the slope of the line should indicate the number of

urea molecules combining with one luciferase molecule (or active site) to produce the inactive luciferase-urea complex.

The data of table 2 have been analyzed in this way and figure 4 shows the result. The points are fitted rather well by a straight line of slope about 1.7. This value might represent a true slope of 2, distorted by experimental errors or, if 1.7 were regarded as a true value, it might represent a complex involving 4 active groups of the enzyme and 7 molecules of urea.

In view of the very great influence of pH, temperature; etc., upon the effects of urea on proteins, it seems hardly useful to try to visualize the type of complex that would yield a value of 1.7 for the slope in the present experiments.

The most striking thing about the inactivation of luciferase by urea is, as mentioned earlier, the relatively low concentration required to produce the effect. At 25°C. and a pH of 6.8, approximately 50% inactivation of the enzyme occurs in only about 0.6 *M* urea concentration! As mentioned earlier, the effects produced on proteins by urea require concentrations of the order of 6 to 12 times this.

Secondary slow inactivation by urea. In addition to the immediate, reversible inactivation of luciferase by urea, a second type of effect was observed. This is a relatively slow loss of activity of the luciferase (about 5% per hour) when it is allowed to stand in urea solutions of the concentrations used to produce the immediate, reversible inactivation already described. This second type of inactivation has not been studied in any detail but it quite certainly represents a different mechanism than that responsible for the immediate type of inactivation.

Luciferase whose activity has been reduced, reversibly, to 50 or 75% by exposure to 0.6 *M* or to 0.3 *M* urea concentrations, respectively, undergoes a gradual further activity loss and this loss is probably irreversible although it has not been measured quantitatively because of difficulties inherent in the present experimental procedure. This reaction quite possibly represents the type of process ordinarily considered to be pro-

tein denaturation. It might be expected that high concentrations of urea (3 to 6 molar, for example) would cause this inactivation to occur at a fairly rapid rate, although such urea concentrations have not been investigated.[4] The reaction merits further study, particularly in relation to temperature and hydrogen ion concentration.

DISCUSSION AND CONCLUSIONS

It has been demonstrated that purified *Cypridina* luciferase is immediately and reversibly inactivated by urea, the degree of the effect depending upon the urea concentration. Under the conditions of the experiments, pH 6.8 and 26°C., the concentration of urea necessary to cause inactivation is considerably lower than that ordinarily required for the denaturation of proteins. This reversible inactivation of the enzyme can be described by a simple mass law equation.

It might seem, at first thought, in the light of these results, that luciferin (the substrate for this luminescent reaction) or some group on luciferin important for combination with the enzyme, might resemble urea structurally. At the present time, very little is known about the structure of *Cypridina* luciferin and there is no information regarding the chemical nature of the enzyme-substrate compound. It seems unnecessary, however, that the inactivating effect of urea upon luciferase need involve combination at the site normally occupied by the substrate. It is generally believed that urea can affect proteins by interfering with normal hydrogen bonding necessary for the native structure of the molecule. Depending upon the extent of the change produced, such an effect might be reversible or irreversible and, in the case of an enzyme, it seems unnecessary that the exact site of combination with

[4] It was, of course, impossible to study this effect with high concentrations of urea with the present experimental procedure because all activity of the enzyme would have been immediately lost, due to the reversible effect, at urea concentrations greater than about 1.5 molar. A more complicated experimental procedure involving dilution of luciferase-urea mixtures at different times after the enzyme had been exposed to the urea might allow a quantitative separation and study of the reversible and irreversible types of inactivation.

the substrate be involved in order to cause reversible inactivation.

Why the concentration of urea required for inactivation of luciferase is so low, compared with that usually necessary for protein denaturation, is puzzling. In the few instances of enzyme inactivation by urea that we have been able to find, relatively high urea concentrations are involved. It is possible that a wide-spread study of enzyme inactivation by urea might reveal that lower concentrations are required than for denaturation of ordinary proteins. Certainly, relatively minor changes in structure might be expected to lead to loss of catalytic power, as compared with the structural change associated with a decrease in solubility or an increase in viscosity, for example.

The secondary, gradual inactivation of luciferase by urea must involve a different mechanism than that responsible for the instantaneous, reversible effect. Presumably, a relatively major change in the structure of the enzyme may be responsible for the slow loss of activity which occurs when luciferase stands in contact with urea in solution. In the present work the existence, only, of this reaction has been pointed out. A more intensive study of it is contemplated, particularly at several temperatures and hydrogen ion concentrations.

Clark ('45) distinguishes 4 separate reactions when urea is added to a protein solution. First, the urea forms a complex with the protein. Second, the protein molecule undergoes denaturation with a change of its typical biological properties. Third, the molecule's solubility decreases, leading to precipitation and, finally, the molecule may split at high urea concentrations. It seems likely that reactions analogous to the first two may be occurring in the present experiments. The immediate, reversible inactivation of the luciferase may be the result of a complex formation between the enzyme and the urea. The rather good description of the data by a simple mass law equation would support such an interpretation. Gradual denaturation might account for the slow, further

decrease in enzyme activity observed when the luciferase is allowed to stand in contact with urea over a period of hours.

There was no observable precipitation of the enzyme in the present experiments, and certainly the concentration of urea was not as high as that ordinarily required to split proteins.

It is significant that, in the case of the immediate inactivation of luciferase by urea, the total amount of light produced was the same regardless of the concentration of urea present, at least up to 1.5 *M*, the highest concentration studied. Only the rate of light production was affected. This would indicate that the enzyme alone is influenced and that there is no reaction between the urea and the substrate, luciferin. Also, it would make it very doubtful that the urea molecule exerts any quenching effect upon the light-emitting molecule or complex, since if such were the case the total light emitted might be expected to vary depending upon the concentration of urea present.

SUMMARY

The luminescent reaction of *Cypridina* luciferase and luciferin, both considerably purified, has been studied as a function of urea concentration, using a precise photoelectric method of measurement.

Two distinct effects of urea upon luciferase were observed. One was an immediate, reversible inactivation of the enzyme; the per cent inactivation increasing with increasing urea concentration. This effect occurred at concentrations of urea of the order of 0.1 to 1.5 *M*. The second effect involved a gradual inactivation of the luciferase and appeared to be irreversible. Higher concentrations of urea were necessary for this effect and it may be comparable with protein denaturations by urea.

The immediate, reversible inactivation of luciferase by urea is describable by a simple mass law equation, which yields a value of 1.7 urea molecules per active catalytic site in forming the hypothetical inactive urea-luciferase complex.

The luciferin is apparently unaffected by urea, at least at the concentrations studied.

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LUMINESCENCE OF CYPRIDINA LUCIFERIN WITHOUT LUCIFERASE TOGETHER WITH AN APPRAISAL OF THE TERM LUCIFERIN [1]

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Although luminescence of Cypridina luciferin by oxidants when dissolved in ethyl alcohol has been observed, no conditions have been previously found under which Cypridina luciferin will emit light in aqueous solutions in the absence of Cypridina luciferase. All attempts to observe luminescence during rapid spontaneous oxidation in water at high temperature or in presence of oxidizing agents from all positions on the redox scale have been in vain (Harvey, '28). In view of the progress in purification of Cypridina luciferin which has been effected in recent years, the action of the various oxidants studied in 1928 has been reinvestigated with luciferin [2] prepared by Anderson's ('35) two cycle benzoylation procedure. The following oxidants were tested as solids or in dilute aqueous solution added to the aqueous luciferin, both without and with 2% H_2O_2: $KMnO_4$, $K_2Cr_2O_7$, $K_3Fe(CN)_6$, Ca hypochlorite, K persulphate, MnO_2, BaO_2, 1% osmic acid, platinized asbestos, cytochrome C (Sigma) and hemin (Nutritional Biochemicals). These oxidants were chosen because they are effective, either alone or with H_2O_2, in causing chemiluminescence of various organic compounds, but in no case did light appear with Cypridina luciferin solutions, although the eye is not as sensitive as modern scintillation counters in detecting low intensity emission.

[1] This research has been in part supported by a contract between the Office of Naval Research (U. S. Navy) and Princeton University, No. 165–167.

[2] For properties of Cypridina luciferin, see Chase ('48).

In this respect Cypridina luciferin differs from crude Pholas luciferin, which emits a readily visible light when treated with $KMnO_4$, BaO_2, and other oxidizing agents (Dubois, '14), a property of hot water Pholas extracts which has been confirmed by Harvey in unpublished observations.

Recently, however, luminescence of fairly concentrated aqueous solutions of Cypridina luciferin, prepared by Anderson's method employing two cycles of benzoylation, have been observed to give a flash of light if mixed with 3% H_2O_2, and a second brighter flash when catalase, freshly prepared in crystalline form from beef liver, is added. If catalase is added to luciferin first, no light appears, but a flash of light follows the addition of H_2O_2. A rapid and voluminous evolution of oxygen occurs. A test with luciferase indicates that some luciferin still remains in the final mixture, but the light is not nearly as bright as when luciferase is added to the original luciferin solution.

Concentrated luciferin solutions dissolved in n-butanol and mixed with 3% H_2O_2 emit a faint light, lasting about one-half minute, which becomes brighter on adding catalase. The luminescence is brightest at the surface in the region of the butanol. Butanol (without luciferin) plus H_2O_2 and catalase emits no light, nor does catalase and H_2O_2 alone. Moreover, a concentrated aqueous solution of luciferin allowed to stand for 14 days until all the luciferin has oxidized, emits no light on addition of H_2O_2 and catalase. There is no doubt but that Cypridina luciferin is responsible for the light emission.

However, the intensity of luciferin luminescence with H_2O_2 is far less than with luciferase. When the aqueous luciferin solution described above is diluted with 5 parts of water, no light is to be observed on adding H_2O_2 or H_2O_2 plus catalase, although the light which appears when the diluted luciferin is mixed with luciferase is brilliant. Luminous efficiency must be thousands of times greater in the presence of luciferase than with catalase and H_2O_2.

One non-biological catalyst has been found, which can take the place of catalase in luminescence of concentrated luciferin,

a commercial substance known as catalyst A (Varniton Co.). This material is highly effective in causing emission of light with luminol (3-aminophthalic hydrazide) and H_2O_2. If catalyst A is added to aqueous luciferin solutions, no light is visible, but if followed by 3% H_2O_2, a flash of light of low intensity occurs similar to that excited by catalase and H_2O_2. A minimal luminescence has been observed when ozonized oxygen is passed through concentrated luciferin solutions, although they rapidly lose their ability to luminesce with luciferase, and have presumably been oxidized.

In view of the well known chemiluminescence of many organic compounds in the presence of strong oxidizing agents, the surprising thing is that oxidation of Cypridina luciferin does not result in luminescence more frequently under similar conditions.[3] In this respect Cypridina luciferin may be contrasted with luminol, which emits light in dilute solution as a result of the most varied chemical treatment. The light intensity of luminol is particularly high if, in addition to H_2O_2, a catalyst is also present. It has been known for a long time that heme compounds and peroxidases will excite the luminescence of luminol, as well as procedures in which nascent oxygen forms, for example at anodes during electrolysis, or during ozone decomposition (Harvey, '29). We have recently observed that luminol will not luminesce when mixed with purified catalase, but if H_2O_2 is added, the light is much brighter than that from luminol and H_2O_2 alone, although not as bright as with H_2O_2 and catalyst A. Luminol appears ready to emit light during almost any disturbance and will luminesce under conditions where oxidation is not directly involved. For example if an alkaline luminol solution is made acid with HCl, a flash of light occurs on neutralization, and if the acid solution is now made alkaline again, another flash of light appears. The acid base change

[3] It is possible that concentrated solutions of Cypridina luciferin might luminesce with oxidants other than H_2O_2, but the supply of pure luciferin has been insufficient to carry out systematic tests.

can be repeated several times, with a flash during the pH change.[4]

Luminol also differs from Cypridina luciferin in that luminol luminescence does not require dissolved oxygen. For example, when potassium ferricyanide and luminol solutions are mixed in complete absence of oxygen, a bright light will appear (Harvey, '40, p. 118). Nevertheless a study of luminol, as an organic chemiluminescent substance, can by analogy aid in the analysis of bioluminescent systems.

Perhaps the most striking fact regarding luminol luminescence is the important part which H_2O_2 plays in light production, particularly in the presence of a catalyst. For this reason, the effect of H_2O_2 and catalase on Cypridina luciferin, previously described, is of particular interest. Hydrogen peroxide formation in various oxidative reactions in living cells has been assumed for many years and the widespread distribution of catalase in the animal and vegetable kingdoms has been interpreted as a means to prevent the accumulation of H_2O_2. Its formation in luminous reactions is a possibility, although no direct evidence is available. Many years ago [4] the effect of H_2O_2 was tested on the luminescence of Cypridina luciferin-luciferase mixtures, using crude solutions of these substances. There was no increased light emission but only a gradual diminution in light intensity which could possibly be attributed to increased non-luminous oxidation of luciferin proceeding simultaneously with the light emitting oxidation. The experiment has recently been repeated, using pure H_2O_2 and purified luciferin and luciferase, with the same result. A weak concentration of H_2O_2 will not increase the light intensity of a glowing luciferin-luciferase mixture, but when enough H_2O_2 has been added to have any effect (about 1 part in 600), the result is always decrease in light intensity, as compared with a control. It therefore seems unlikely that H_2O_2 plays a part in Cypridina luminescence.

[4] Unpublished experiments of 1928–29.

A similar experiment with pure H_2O_2 and a suspension of luminous bacteria (Achromobacter fischeri) has also indicated that minute amounts of H_2O_2 do not increase bacterial light intensity, and higher concentrations (1:300,000) decrease luminescence slightly, with a rapid reversal (15 seconds). Still higher concentrations reduce the luminescence still more, with a slower return to the original brightness. It therefore takes 1000 times less H_2O_2 to affect the luminous bacterial light than that of the Cypridina luciferin-luciferase reaction.

The slow return of light intensity of luminous bacteria can be greatly accelerated by adding catalase to the suspension, presumably by decomposition of the added H_2O_2. Catalase alone does not affect the light intensity of luminous bacteria. It would be of considerable interest to make quantitative studies on the effect of H_2O_2 on various luminous bacterial species, especially since van Schouwenburg ('40) has shown that the catalase content of different species varies considerably.

There is much evidence to indicate that the luciferins of a number of different luminous animals are quite different substances chemically, and have arisen independently during the course of evolution (Harvey, '53). Enough is known of the chemistry of luminescent systems in the fire-fly and in bacteria to compare them with Cypridina. For example, the luminescence system of Cypridina, in addition to oxygen, requires only the presence of luciferin and luciferase, whereas in the case of the fire-fly, luciferin, luciferase, adenosine triphosphate (ATP) and Mg ion are all essential. The influence of phosphate can be demonstrated by the reappearance of light when ATP is added to a water extract of fire-fly lanterns allowed to stand until the luminescence has disappeared (McElroy, '47). The ATP is not all used up, but is immobilized, and can be displaced by Na pyrophosphate and organic pyrophosphates, resulting in a second luminescence (McElroy, '51).

If similar experiments are carried out with water extracts of dried Cypridinas,[5] no light is to be observed on adding ATP, nor has it been possible to obtain light on adding other phosphates, representing both high and low energy samples. The following have been tested: adenosine diphosphate (ADP, Sigma); diphosphopyridine nucleotide (DPN, Sigma); triphosphopyridine nucleotide (TPN, Sigma); riboflavin phosphate (FMN, Sigma); creatine phosphate hydrate (Sigma); Ba inosinetriphosphate (Sigma); p-nitrophenyl phosphate (Sigma); thiamine pyrophosphate (Merck); acetyl phosphate (Nutritional Biochemicals); Na pyrophosphate (Mallinckrodt); Na glycerophosphate (Eastman); Na triphosphate; K glucose-1-phosphate, Ba glucose-6-phosphate and Ba fructose-6-phosphate (of Nutritional Biochemicals); hexose diphosphate (Schwarz); Na phosphates (Na_3PO_4, NaH_2PO_4); Na metaphosphate; Na hexametaphosphate; Na hypophosphate; Na phosphite and Na hypophosphite. Phosphate does not appear to be involved in the light production of Cypridina.

The recent demonstration of luminescence in cell free extracts of luminous bacteria (Strehler and Cormier, '53; Cormier and Strehler, '53; McElroy, Hastings, Sonnenfeld and Coulombre, '53) on addition of flavins, coenzymes and long chain aliphatic aldehydes (the kidney cortex factor, KCF) and the designation of riboflavin phosphate as bacterial luciferin (Strehler, Harvey, Chang and Cormier, '54) has led us to test the new group of bacterial accessory substances on the Cypridina luciferin-luciferase system. Every experiment has given negative results. No light has been observed which can be attributed to reduced riboflavin phosphate (FMN · H_2), reduced riboflavin, reduced diphosphopyridine nucleotide (DPN · H_2) or such mixtures as DPN + malate, or DPN · H_2 + FMN, with or without decaldehyde, when added to dark crude Cypridina extracts, although these substances may par-

[5] For the effect of ATP on dark extracts of fresh luminous tissue of a variety of organisms, see Harvey and Haneda ('52) and Haneda and Harvey ('54).

tially reduce oxidized luciferin whose reoxidation emits a weak light.

All the above substances or mixtures will evoke light when added to dark luminous bacterial extracts. Neither decaldehyde nor palmitaldehyde in small amounts added to a glowing mixture of Cypridina luciferin and luciferase, will increase the light intensity; dodecaldehyde added to saturation slowly decreases the luminescence as compared with a control. It has also been determined that no light appears when the *reduced form* of such biological substances as thiamine HCl (Nutrit. Biochem.), thiamine pyrophosphate (cocarboxylase, Merck), TPN (Sigma), glutathione (General Biochemicals), alloxan (Eastman) and cytochrome C (Sigma) are added to dark aqueous Cypridina extracts.

All the above experiments point to the conclusion that luminescence in Cypridina, in the fire-fly, and in luminous bacteria differ fundamentally in the chemical systems involved. Crude luciferin extracts of one group of luminous organisms will not produce light with the crude luciferase extracts of another group, unless very closely related, for example, families of the same order (Harvey, '22, '26). Recent work with purified luciferin and luciferase preparations has confirmed these findings. Purified fire-fly luciferin and purified Cypridina luciferase emit no light when mixed, even though ATP and Mg salts are also present, and the same is true for purified fire-fly luciferase and purified Cypridina luciferin (McElroy and Chase, '51).

The experiments mentioned in this paper indicate that luciferin and luciferase of the bacterial luminescent system and the Cypridina system, cannot replace each other. Cypridina luciferin will not luminesce when added to bacterial extracts and decaldehyde. The bacterial studies have also raised the important question as to which substance should be regarded as luciferin, especially as it seems highly probable that the luciferins from different groups of luminous organisms are chemically quite different. Although light appears

in bacterial extracts (containing bacterial luciferase) when reduced riboflavin phosphate is added, the luminescence is many times brighter if an aliphatic long chain aldehyde is also present. Is the aldehyde or the flavin to be designated as "luciferin"? The answer requires a general definition of the word luciferin.

Dubois (1887) originally gave the name "luciférine" to the heat stable, dialyzable, oxidizable substance in a hot water extract of the light organ of the elaterid beetle, Pyrophorus, or the mollusc, Pholas dactylus. He found that luminescence appeared when luciferin was mixed with a dark cold water extract of the light organs of the above animals. The cold water extracts contained "luciférase," a thermolabile non-dialyzable enzyme, whose catalytic oxidation of luciferin resulted in light emission. A similar "luciferin-luciferase reaction" was subsequently demonstrated in North American lampyrid fire-flies (Harvey, '16), in the ostracod crustacean, Cypridina (Harvey, '17), the marine fire-worm of Bermuda, Odontosyllis enopla (Harvey, '22), and the shrimp, Systellaspis debilis (Harvey, '31). The reaction has been reported in the fresh water limpet, Latia neritoides (Bowden, '50). A large number of other luminous organisms failed to exhibit a luciferin-luciferase reaction (Harvey, '22, '26, '31). The explanation of such negative results may lie in destruction of luciferin or luciferase during preparation of extracts or in loss of other accessory factors necessary for light production.

It is no longer sufficient to claim that luciferin is present in a boiled extract of luminous tissue whereas the dark cold water extract contains luciferase. McElroy has demonstrated that dark cold water extracts of fire-fly (Photinus pyralis) lanterns emit no light when purified pyralis luciferin is added, but do luminesce with ATP. Therefore the cold water extract (luciferase) lacks ATP instead of luciferin. ATP is the limiting factor under these conditions.

Rather than placing the emphasis on heat stability or dialyzability or even oxidizability,[6] as has been done previously, light emission should be the criterion. In the case of luminous organisms requiring dissolved molecular oxygen for luminescence, luciferin may properly be defined as the oxidizable substance supplying molecules capable of absorbing enough excess energy to emit in the visible region. Such a definition implies that some form of luciferin molecule — either free base or acid, either dissociated anion or cation, in reduced or oxidized form, either free or combined with protein, like a prosthetic enzyme group — can pick up the energy of the oxidative reaction in which it is involved. Such a definition does not mean that luciferin is the same substance in different luminous animals, nor does it necessarily designate luciferin molecules themselves as the ones which emit, but it does imply that a related molecule, such as a luciferin-luciferase combination, or an oxidized luciferin molecule, or a molecule of an intermediate step, is the emitter. The molecule actually emitting might be referred to as the photogen. The luciferin-luciferase reaction is comparable to the lophin and blood plus H_2O_2 luminescence of Ville and Derrien ('13), or the pyrogallol and potato juice plus H_2O_2 luminescence (Harvey, '16), or to luminol and the many catalysts plus H_2O_2 (or oxidants) which excite chemiluminescence of this compound.

It has long been recognized that a substance, whose molecules are readily excited to fluoresce by the energy of radiation, is most likely to be chemiluminescent from the energy of a chemical reaction. The well known chemiluminescent materials [metal prophyrins, phthalhydrazids (luminol), lophins, acridinium derivatives (lucigenin), and aesculin] are all fluorescent, either as ions or molecules in aqueous solution. Moreover fluorescence of the light organs of luminous animals is widespread (Harvey, '26). Therefore it seems most logical to regard the reduced form of the fluorescent flavin as bacterial luciferin rather than the non-fluorescent

[6] Dissolved oxygen is not necessary in the case of ctenophores, certain medusae, and radiolarians (Harvey, '26).

aldehyde. Although riboflavin and FMN are not fluorescent in the reduced form, the oxidized flavins fluoresce yellow green over a wide pH range, from pH 1 to 11. An analogous situation is to be observed among the ctenophores, where a striking phenomenon is the fluorescence of the luminous organ after, but not before bioluminescence has occurred, as if the final product of luminescence was a fluorescent molecule (Harvey, '25). In the case of luminol, chemiluminescence cannot be demonstrated in an acid solution whose blue fluorescence is marked; in alkaline solution chemiluminescence but not fluorescence is readily excited.

Fire-fly luciferin is known to be highly fluorescent and the question arises whether any form of Cypridina luciferin is also fluorescent. Previous statements on the fluorescence of Cypridina luciferin have all been in the negative. Certainly this substance does not exhibit the marked fluorescence characteristic of flavins, quinine sulphate, aesculin, and various dyes, but a concentrated solution of luciferin in water is somewhat fluorescent. For these observations the Anderson purified material was paper chromatographed, using the top layer of a mixture of ethyl acetate, ethanol and water in the proportions 5:2:3 by volume, at pH 6 to 7, in a cold room at 2.5°C. After 4 hours the paper exhibits a band of yellow material with R_F about 0.6 and a brownish region at the solvent front. The yellow band (R_F 0.6) is the only part of the paper showing a bright luminescence when sprayed with luciferase. It is also brightly yellow fluorescent in the ultraviolet light from a mineralite lamp and less so in ultraviolet wave-lengths from a mercury arc with Wood's filter, or in the light from a "purple ultra" incandescent bulb. The only other fluorescent region of the paper is at R_F of about 0.3, emitting with a blue color.

Paper electrophoresis of the Anderson purified Cypridina luciferin has also revealed a yellow and a blue fluorescent region. The yellow fluorescent region has a yellow color and corresponds to luciferin, giving light with luciferase.

When the luciferin region of the paper chromatogram is eluted with methanol, the solvent removed in vacuo and the residue dissolved in a small amount of 0.1 N HCl, this solution exhibits a yellow fluorescence under the mineralite lamp. In the "purple ultra" light, the fluorescence appears greenish. If the solution of luciferin is diluted with 5 parts of water, no fluorescence is visible, and if the original luciferin solution is examined in a beam of white light, such as readily excites the fluorescence of flavin or quinine SO_4, no fluorescence can be observed. All these experiments indicate that the fluorescence intensity must be low.

When the luciferin in 0.1 N HCl is neutralized with NaOH, the yellow fluorescence remains, and in 0.1 N NaOH becomes at first brighter and then less intense over a course of many days, as the luciferin oxidizes spontaneously. The oxidized luciferin, which gives no light with luciferase, is also very weakly fluorescent.

Spectral absorption studies of the paper chromatographed and paper electrophoresed Cypridina luciferin have been carried out with the collaboration of Dr. A. M. Chase of Princeton University and will be reported in a later paper. Suffice it to say that in 0.1 N HCl they reveal an absorption peak at 265 and a shoulder at 305 mμ; in alkali a peak at 265 and 385 mμ, the latter peak stabilizing at 357 mμ. These absorption bands could be the wave-lengths exciting luminescence. Concentrated methanol and butanol solutions of luciferin are also yellow fluorescent. On spontaneous oxidation of Cypridina luciferin in the air, the yellow color disappears and the yellow fluorescence decreases.

It is clear that Cypridina luciferin does exhibit fluorescence, although it is far less bright than the fluorescence of many other chemiluminescent substances. Perhaps this is because the fluorescent group is bound with a large number of amino-acids to make up the polypeptid structure, which appears to be characteristic of Cypridina luciferin (Mason, '52). Such combinations have a profound effect on fluorescence and can completely suppress the fluorescence in the case of flavins

which combine with proteins to form certain of the yellow enzymes. A detailed study of the quenching of fluorescence of some of the flavins has been made by Weber ('50).

If luciferin is to be defined as the substance which supplies molecules that in one form or another can be excited to emit as a result of electron transitions resulting from the energy of a chemical reaction, present knowledge points to riboflavin phosphate in bacteria and the compounds now called luciferin (composition unknown) in both the fire-fly and in Cypridina as fitting this designation. Although chemically distinct, they are all fluorescent, indicating the presence of luminophore groups, and in one form or another can all be oxidized with light production by H_2O_2.[7] Finally they all luminesce most intensely in presence of their own specific luciferase, provided dissolved oxygen is present. The fact that they are also heat stable (in absence of oxygen) and dialyzable is of secondary importance. Adenosine triphosphate and Mg in the case of the fire-fly, and the long chain aliphatic aldehyde in the case of luminous bacteria, do not correspond to the above definition, and might be regarded as accessory substances. Possibly aldehyde acts by affecting the quantum efficiency of the emission. Such action is the converse of a quencher in luminescence reactions.

SUMMARY

Momentary low intensity luminescence of purified Cypridina luciferin has been observed in presence of hydrogen peroxide. The light becomes brighter if pure catalase or "catalyst A" are also present. Catalyst A is particularly effective in exciting luminescence of 3-aminophthalic hydrazid (luminol). The Cypridina luminescence reaction is compared with that of luminol chemiluminescence by oxidants.

Hydrogen peroxide added to a glowing mixture of purified Cypridina luciferin and luciferase reduces light intensity in the lowest concentration which has any effect, probably a result of oxidation of luciferin. One thousand times less H_2O_2 is necessary to inhibit bacterial luminescence.

[7] See Strehler and Shoup ('53) for riboflavin luminescence with H_2O_2. Doctor McElroy has informed us that fire-fly luciferin will luminesce with H_2O_2.

The various substances which cause light emission in cell-free bacterial extracts (flavins, pyridine nucleotides, long chain aliphatic aldehydes), or those involved in fire-fly luminescence (bivalent cations, various phosphate compounds) play no part in the extracellular Cypridina luminescence system.

Definition of the term, luciferin, is discussed and the suggestion made that for organisms whose luminescence requires oxygen, luciferin is that oxidizable substance supplying molecules or ions which in one form or other (in reduced or oxidized state, free or combined with luciferase) emit light as a result of electron transitions from the energy of a chemical reaction.

It is to be expected that luciferin molecules in one form or other will be fluorescent. A low intensity yellow fluorescence of Cypridina luciferin, highly purified by paper chromatography, has been observed in ultraviolet light.

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PHOSPHATE EXCHANGE IN NERVE[1,2]

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FIVE FIGURES

The uptake of phosphate by muscle (Sacks '44) and by yeast (Mullins '42) has been shown to be a process whose rate is dependent upon metabolic activity. It has been possible to demonstrate (Rothstein and Meier '48) that in the case of yeast, phosphate transfer across the cell membrane is effected by enzymes located at the cell surface. Further, the transfer of phosphate across the red cell membrane is dependent upon metabolism (Eisenman et al., '40) and phosphate transfer in microöganisms can be inhibited strongly by 2.5 mM azide (Spiegelman and Kamen '48). There seems no doubt that a small inward leakage of phosphate can occur (Furchgott and Shorr '43) but this is only a few percent of

[1] Aided in part by a grant (B-139) from the National Institute for Neurological Diseases and Blindness, Bethesda, Md. Isotopes used in these experiments were obtained on allocation from the U. S. Atomic Energy Commission.

[2] Since the preparation of this manuscript, two papers have appeared that deal with the penetration of P^{32} into nervous tissue. In the first, (Abood, L. G. and R. W. Gerard, Fed. Proc. 13, 1, 1954) a definite decrease in the rate of phosphorylation in both brain and nerve during activity has been observed. Further, phosphate turnover in both the ATP and CrP fractions of both tissues when stimulated was decreased over control values. In a second paper, (Oomura, Y. and Inanaga, K., Japanese J. Physiol. 3, 119, 1953) frog sciatic nerve was equilibrated with about isosmotic sodium phosphate. Disregarding the fact that Ca^{++} must have thus been eliminated from the Ringer solution, the authors' conclusion that phosphate uptake takes place as a physical process is understandable because with such low specific activites of phosphate as were used, the process being followed was undoubtedly the equilibration of P^{32} with the nerve extracellular space.

normal phosphate transfer rates. When radioactive phosphate is allowed to enter a muscle, P^{32} appears first in the creatine phosphate and ATP fractions of the intracellular phosphate, and only after about 4 hours do appreciable amounts of P^{32} labelled orthophosphate appear in the cell. Presumably this labelled orthophosphate is derived from the breakdown of labelled esterified phosphate in the muscle. Because of the absence of any detailed information on the rate of transfer of phosphate in nerve, where it is possible to separate the phenomenon of excitation from that of contraction, we have investigated the uptake of phosphate by frog sciatic nerve under a variety of experimental conditions.

METHODS

Sciatic nerves from R. pipiens were dissected and kept in a small volume of Ringer solution before being used in these experiments. Two arrangements were used for the equilibration of nerves with radioactive phosphate solution. In the first, a nerve was placed in 1 ml of Ringer solution containing P^{32} as phosphate for the required length of time, and then the nerve was washed, dried, and its radioactivity measured with a Geiger-Muller counter. In the second arrangement, shown in figure 1, it was possible to count the radioactivity of nerve plus a small volume of solution whenever a count was desired. Here the nerve was introduced into a closely fitting thin-walled glass tube containing sealed-in platinum wires for stimulating and recording. Radioactive phosphate solution was pulled slowly through this tube at a rate of about 1 ml/min., and a counting tube, shielded from all but the direct radiation coming from the glass tube, measured the radioactivity. Such an arrangement would not be possible were it not for the fact that phosphate is accumulated by the nerve to a very considerable extent over its concentration in the external solution. Even quite early in a run, the ratio of P^{32} inside to out was about 10:1. Since the nerve usually occupied about 80% of the total volume of the tube, the contribution of the P^{32} outside the nerve to the total count was only

about 2%. The extracellular space of a frog sciatic nerve is about 50%, so that with a ten-fold intracellular accumulation of P^{32}, the extracellular space would add another 4% to the count of P^{32} outside the nerve. We have also noted increases in the weight of nerves immersed in Ringer solution for long periods of time. This weight increase involves a volume increase and hence would constitute an error. Over a period of two hours, however, volume changes in nerve

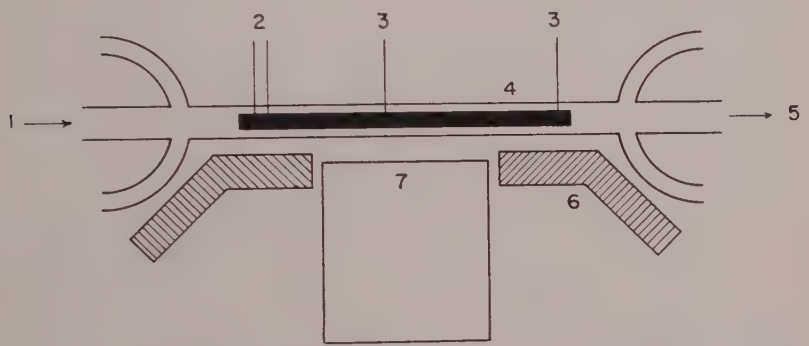

Fig. 1 A tube for the measurement of P^{32} in nerves. 1, inflow for P^{32} Ringer solution. 2, stimulating electrodes. 3, recording electrodes. 4, thin-walled glass tube. 5, outflow to syringe pump. 6, lead shielding. 7, counting tube.

were entirely negligible. The Ringer solutions in all cases were saturated with either O_2 or $O_2 + 5\%$ CO_2, and the pH was 7.0. The temperature varied between 20–23°C. for all experiments except those carried out at 2°.

RESULTS

The uptake of labelled phosphate by frog sciatic nerve proceeds in an approximately exponential manner with time at 20°C. and in the presence of oxygen as shown in figure 2. On the other hand, if metabolism is reduced, by reducing the temperature to 2°C. or in the presence of nitrogen, or of azide, an inhibitor of phosphorylation at this concentration, then phosphate uptake is negligible. These results were obtained using a phosphate concentration of 0.1 mM/L. Be-

cause the P^{32} used in these experiments was essentially carrier-free, measurements of phosphate uptake could be made at very low concentrations, and as the data in figure 3 shows, the rate of uptake of phosphate is almost maximal at a concentration of 0.5 mM/L. Because of many references to the effect of phosphate on cells, it is worthwhile to examine the

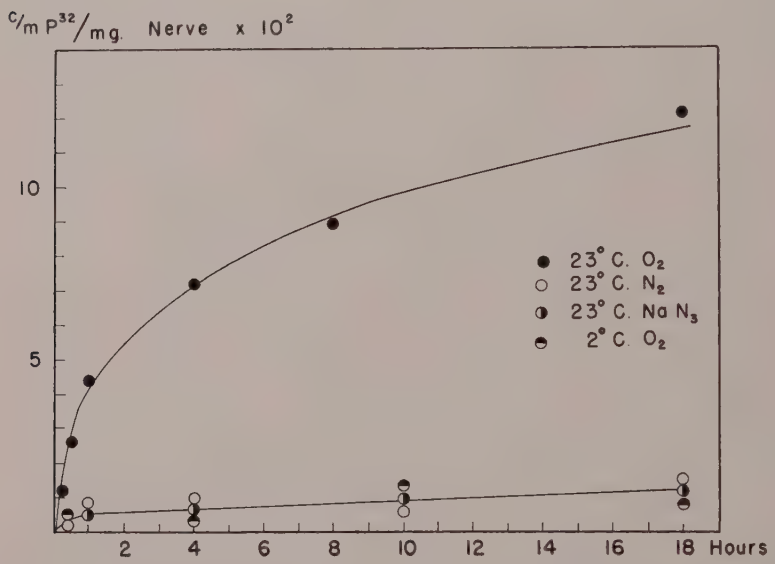

Fig. 2 The uptake of P^{32} by frog sciatic nerve. The upper curve shows the uptake of P^{32} (as counts/min/mg nerve vs. time) at 22°C. in the presence of O_2. The lower curve represents P^{32} uptake while metabolism is inhibited in some way. The external phosphate concentration was 0.1 mM/L in all cases, and the Ringer solution has a count of 200 c/m/mg.

concentrations permissible in Ringer solution if Ca^{++} is not to be disturbed. Phosphoric acid is a relatively strong acid with a $K_1 = 1.1 \times 10^{-2}$. At pH 7, this means that the ratio of ionized to unionized molecules will be 10^5, or that the material is, for all practical purposes, entirely ionized. The $K_2 = 7.5 \times 10^{-8}$, so that at pH 7, the phosphate consists of about an equimolal mixture of $H_2PO_4^-$ and $HPO_4^=$. Now the solubility of $Ca(H_2PO_4)_2$ is 71 mM/L but the solubility of

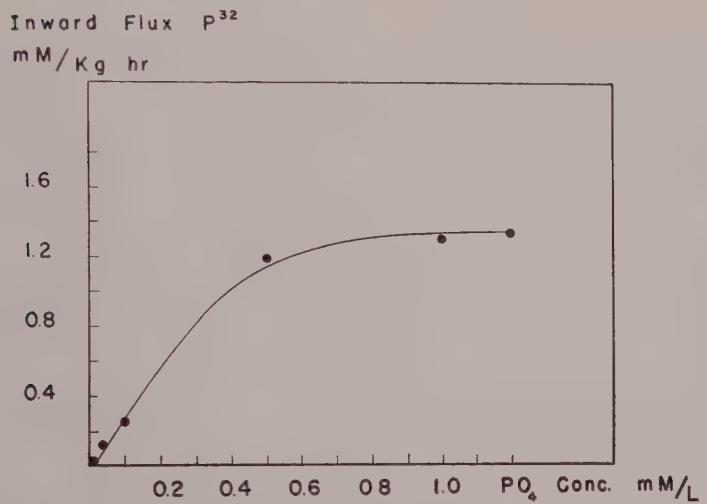

Fig. 3 The inward flux of P^{32} in mM/Kg nerve hr. is plotted against PO_4 concentration in Ringer solution. Rates are derived from the initial slope of P^{32} uptake curves at various concentrations.

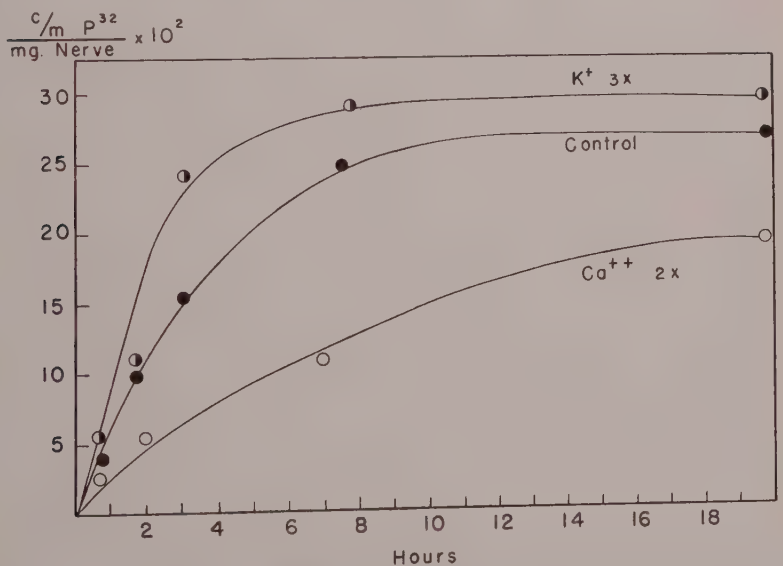

Fig. 4 The effect of changes in the composition of Ringer solution on the uptake of P^{32} by frog sciatic nerve at 22°C. in O_2 is shown by curves labelled Ca^{++} 2 × (3.6 mM) and K^+ 3 × (6.0 mM). The control curve represents uptake in normal Ringer solution. External phosphate concentration is 0.5 mM/L and the external solution gave a count of 600 c/m/mg.

$CaHPO_4$ is only 1.2 mM/L. The solubility product is $(1.2)^2 = 1.4$. Hence if Ca^{++} in Ringer is 2.0 mM/L, $HPO_4^{=}$ cannot be more than 0.7 mM/L, or total phosphate more than 1.4 mM/L. We have investigated the effect of high Ca^{++} as well as the effects of high K^+ on the uptake of phosphate and the results are shown in figure 4. The effects of zero Ca^{++}, and of citrate, are so variable that we have not included them in figure 4. In about half of the nerves tested, zero Ca^{++} Ringer or isotonic citrate greatly increased the rate of uptake, while other nerves that appeared normal in every respect did not take up any appreciable amount of P^{32}. In many ways these results parallel those of Brink ('51) on the changes in oxygen consumption of nerve.

The effects of stimulation on the uptake of P^{32} by nerve are summarized in figure 5. A most surprising finding is that stimulation at 60/sec (supra-maximal for A fibers) apparently blocks the uptake of P^{32}. At the same time, it can be shown that stimulation of a nerve that has accumulated a considerable amount of P^{32}, leads to no change in its P^{32}. This latter finding is more in accord with expectations in that one assumes that upon stimulation the ester phosphate fraction of nerve breaks down and while some orthophosphate escapes, most of it in resynthesized. There remains the possibility that the decrease in P^{32} uptake with stimulation is some sort of stimulus artifact, although when an inexcitable nerve is stimulated, PO_4 uptake is normal. We have repeatedly observed that nerves that could not be stimulated to conduct a propagated disturbance, showed a quite normal rate of uptake of P^{32}. From this standpoint, phosphate uptake is similar to the development of a demarcation potential in that both of these processed can apparently proceed normally whether or not the nerve is excitable. Grande and Richter ('49) have reported that stimulated frog sciatic nerve shows a 200% increase in the rate of exchange of P^{32} as compared with a resting nerve. Their experimental conditions were sufficiently different from ours so that such a finding

is not necessarily a disagreement. Their nerves had the sheath removed, and had already accumulated considerable P^{32}. It has been shown (Mullins and Grenell '52) that sheath removal greatly accelerates the uptake of P^{32}. Presumably this acceleration works both ways, so that PO_4 lost to the extracellular space by stimulation, is not recovered by the nerve during rest, and fibers are not limited in their uptake of P^{32}, by the small amounts available in the extracellular space.

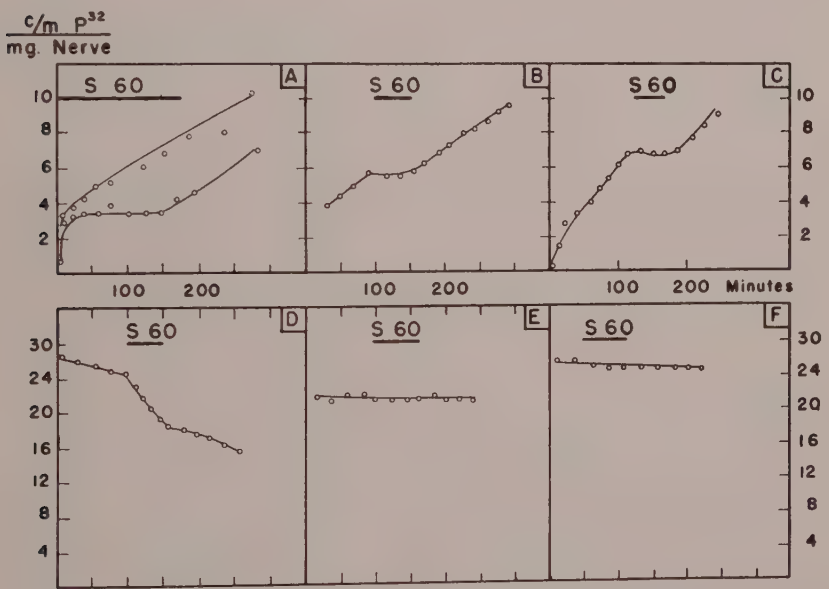

Fig. 5 Ordinates are counts per minute of P^{32} (to be multiplied $\times 10^2$) per milligram of nerve.

(A) The effect of stimulation at 60/sec for a time interval shown by the bar over the curve, on P^{32} uptake by nerve. Upper curve, resting nerve; lower curve, stimulated nerve. External phosphate concentration 0.1 mM/L = 200 c/m/mg.

(B), (C) The effects of stimulation on P^{32} uptake by nerve. Conditions as in (A).

(D) The loss of previously accumulated P^{32} by frog nerve. The nerve after 24 hrs. in P^{32} Ringer was transferred to inactive Ringer containing 0.1 mM/L PO_4 at the start of the experiment. Stimulation was as indicated above.

(E), (F) After 24 hours in P^{32} Ringer, nerves were stimulated in P^{32} Ringer solution (0.1 mM/L). Other conditions as above.

DISCUSSION

Peripheral nerve appears to have about the same creatine content as muscle, Gerard '32 gives 0.16% which is 24 mM/kg fiber water, assuming a 50% extracellular space, while Eggleton (see Boyle and Conway, '41) gives 25 mM/kg muscle which is 38.5 mM/kg fiber water. ATP is only about 15% of this figure or 5 mM/kg for muscle. Figures for orthophosphate in tissue are quite variable, undoubtedly because the method of sampling the tissue determines to a large extent just how much ATP and CrP break down before the analysis is performed and such breakdown results in high figures for PO_4. In a detailed analysis of phosphate fractions of spinal cord tissue Abood et al. '52 show that CrP is about 30% of the total acid soluble phosphate, and orthophosphate and glucose-PO_4 each about 15%. The available evidence from muscle and yeast studies would suggest that PO_4 is taken into the cell as a phosphorylated compound (Sacks, '48). Mommaerts ('50) indicates that ATP is breaking down in muscle during rest to the extent of about 2% of the total amount per minute. This gives one an idea of the probable equilibration time for externally supplied phosphate, provided its penetration depends upon such breakdown rates. Indeed, Abood et al. ('52) find that only 30 minutes are required for the equilibration of P^{32} with ATP in peripheral nerve. We have used nerves weighing between 20 and 50 mg, these would have approximately 4 mM/kg (8 mM/kg fiber water) CrP. The observed inward flux of PO_4 of 1.2 mM/kg hr. is about 25% of the total CrP present per hour. Our data are consistent with the view that phosphate enters nerve fibers as a phosphorylated complex of some sort, and that this complex is dissociated during both rest and activity with the consequent escape of some of the phosphate to the outside. While in yeast, phosphate transfer can be limited by limiting the concentration of glucose in the external solution, frog nerve is apparently not sensitive to external glucose concentrations. The decreased rate of P^{32} uptake by stimulated

nerve may be compared with the observation of Abood and Gerard ('52) that there is a decreased P^{32} turnover in the brains of mice subject to audiogenic siezures. These authors also cite evidence for a decreased turnover of P^{32} during nervous activity. A possible explanation of this effect might be that materials leaking out of stimulated nerves combine with the extracellular phosphate, making it thus unavailable for penetration.

I am indebted to Dr. D. W. Bronk for making available facilities for this work, and to Dr. F. Brink for suggesting this problem.

SUMMARY

The uptake of P^{32} by frog sciatic nerve is blocked by oxygen lack, low temperatures, low concentrations of azide, and increased Ca^{++} concentrations in Ringer solution. Stimulation decreased the uptake of P^{32} but did not affect the total content of previously accumulated P^{32}.

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POTASSIUM AND SODIUM MOVEMENTS IN RABBIT POLYMORPHONUCLEAR LEUKOCYTES

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EIGHT FIGURES

Studies on the electrolyte pattern in leukocytes have been limited. Hamburger and Van der Schroef ('02) had demonstrated the permeability of leukocytes to anions and their apparent impermeability to cations. Endres and Herget ('29) reported on the electrolyte composition in horse leukocytes. Fleischmann ('29) assumed an impermeability of horse leukocytes to Ba^{++} and Ca^{++} ions on the basis that these ions did not reduce the respiratory rate of intact cells, but did cause a marked reduction in cells whose membranes had been injured by freezing and thawing. Shapiro and Parpart ('37) measured the permeability of both human and rabbit leukocytes to water. Pulver and Verzar ('41) studied potassium movement in horse leukocytes in connection with glucose utilization. Recently, Wilson and Manery ('49) have determined the K^+, Na^+, and Cl^- content of rabbit leukocytes, and studied some of the permeability characteristics of these ions. The author ('52) has determined the K and Na content in dogfish leukocytes obtained from the peripheral circulation. Vallee and Gibson ('48) have claimed the presence of zinc in human leukocytes.

[1] Fellow, Atomic Energy Commission, 1951–1953.

[2] This work was in part supported by the Eugene Higgins Fund of Princeton University.

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It would be of interest to determine to what extent the internal K^+-Na^+ content of the rabbit leukocyte would change under *in vitro* conditions, and secondly, what factors were acting to limit or accelerate these changes.

PROCEDURES

Collection. Cells were obtained by a slight modification of Hamburger's classical technique ('27). From 300–500 cm^3 of a sterile 0.95% saline solution were introduced intraperitoneally. For the experiments with glucose, a mixture of 0.95% saline and 0.11 M NaH_2PO_4-0.11 M Na_2HPO_4 buffer (pH 7.2) in a ratio of one part of buffer to 9 parts of saline solution was used. After 12 to 18 hours, approximately 300 cm^3 of this same solution were injected as a lavage. A few minutes later, cells were removed by trochar into a collecting flask, containing a few cubic centimeters of heparin. For long term experiments (24 hours), aseptic conditions were observed. Usually, the cell suspensions from three to six rabbits were pooled. The type of cell obtained was almost exclusively polymorphonuclear. Red cells never exceeded 6% of the total number of cells. Check experiments indicated that this number did not measurably affect the K^+ and Na^+ determinations.

Per cent water. Aliquot samples of 40–50 cm^3 were taken from mixed cell suspensions and the cells were separated either by the separator head technique or first centrifuged in 50 cm^3 centrifuge tubes and then transferred to small tubes for packing in the air turbine (Parpart and Green, '51). Wet weights were obtained, after which the cells were frozen, and then dried under high vacuum. Dry weights were obtained by difference, and the per cent water calculated.

K^+ and Na^+ analyses. Wet weights taken for analysis usually ranged from 0.05 to 0.15 gm. Duplicate aliquot samples were taken from the suspension for each analysis. The cells were packed in the air turbine and after obtaining the wet weight, were then transferred to a known volume of distilled water, shaken intermittently for at least 30 minutes, and the cell debris centrifuged. Analyses were made on the super-

natant, using a Perkin-Elmer flame photometer; in later experiments, a Beckmann type was used.

pH readings. These were made electrometrically, using a Cambridge glass electrode pH meter.

Glucose determinations. Aliquots of 1 cm³ of cell-free exudate were taken for glucose analysis, using the Nelson method ('44), with some modification for Beckmann spectrophotometric analyses. Glucose content is usually determined by its ability to reduce cupric ion. In this method the amount

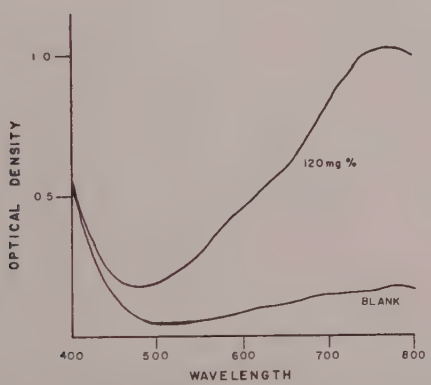

Fig. 1 Absorption spectra for colored solutions produced by a glucose standard of 120 mg % and a blank with no glucose, according to the Nelson method. Measured with a Cary recording spectrophotometer.

of cuprous ion formed is measured by its reaction with an arseno-molybdate chromagen to form a colored complex. This is determined colorimetrically. Although a wavelength of 500 mμ was suggested in the Nelson paper, it was found that a wavelength of 750 mμ gave greater sensitivity. This is indicated in figure 1. The color developed with a glucose standard of 120 mg % is contrasted with that of a blank by comparing their absorption spectra. The range of readings made at 750 mμ is 5.6 times that at 500 mμ and 1.6 that at 660 mμ, which was stated to be the wavelength of maximum sensitivity for the chromagen. Figure 2 shows a typical calibration curve at 750 mμ.

RESULTS

Per cent water. In order to interpret changes in the electrolytes, one must have information about their concentrations. This requires a knowledge of the amount of intracellular water present. Table 1 shows the per cent water content of leukocytes under various experimental conditions. The time periods include the time necessary for collection and transfer to a constant temperature bath. This time never exceeded one hour and averaged about 45 minutes.

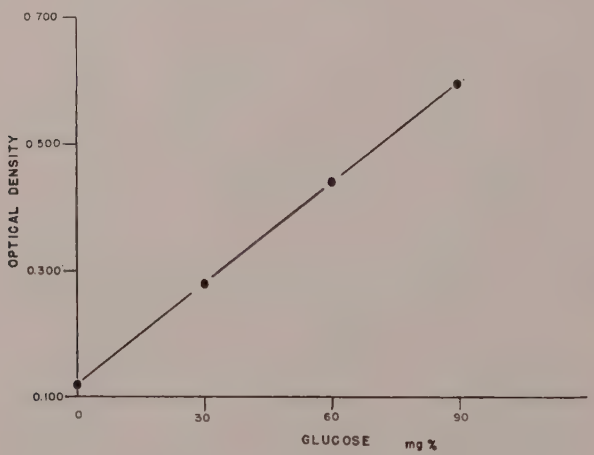

Fig. 2 Calibration curve for glucose determinations.

Over a period of 7 hours, there was no significant change in water content under any of the conditions tested. Over a 24-hour period, the greatest change was a 6% increase in water content.

Although not indicated in table 1, some of the experiments were of the type where the cells were packed by the separator head technique, while others involved initial packing in large centrifuge tubes and then transfer to smaller tubes for packing in the air turbine. There was no significant difference in the per cent water content of cells obtained by the two methods. This would indicate that there was no selection

involved in the separator head technique, so far as the cell population was concerned. The overall mean water content was 79.0 ± 1.14 for cells obtained by saline injection and lavage. This agrees fairly well with a value of 79.25 ± 0.65 obtained by Wilson and Manery ('49) for cells exposed to a modified Ringers solution for 45 minutes at 37°C.

TABLE 1

Per cent water content of rabbit leukocytes under different experimental conditions

SYSTEM	HOURS AFTER REMOVAL							
	1	2	3	4	5	6	7	AVG.
37°C. unbuffered: No glucose added.	78.7 ± 0.77 (19)	79.1 ± 1.59 (4)	78.2 ± 0.78 (2)	79.0 ± 0.64 (2)	79.5 ± 1.67 (6)	80.5 ± 1.00 (3)	79.1 ± 1.21 (3)	79.0 ± 1.14 (39)
37°C. unbuffered: 100–300 mg % glucose	79.7 ± 0.3 (3)	80.0 ± 1.87 (3)	78.0 (1)	77.7 (1)	79.2 (1)	78.7 (1)	77.7 ± 1.2 (3)	78.9 ± 1.38 (13)

	"0" TIME	24 HOURS
Injection and lavage with saline-PO_4. System composed of 1 part exudate to 1 part undiluted buffer at pH 7.2, with glucose.	75.7	78.4
Injection and lavage with saline-PO_4. Exudate used as obtained, with and without added glucose.	75.0 76.2	73.5 77.4

( ) indicated number of samples.

The presence of added glucose does not alter the water content significantly. However, mixing exudates with phosphate buffer solutions does lower the water content. The change in water content is believed to be due to a penetration of phosphate ion into the cell in exchange for chloride ion with a net loss of osmotically active material. Further work is being carried out to verify this hypothesis.

Base line values for K^+ and Na^+. Table 2 shows the average K^+ and Na^+ content of rabbit polymorphonuclear leukocytes

obtained and analyzed by the methods indicated above. Comparisons are made with values obtained by Wilson and Manery ('49) who used a complete ashing technique. Also included are values for other animals as reported in the literature.

The fact that values for K^+ or Na^+ do not differ significantly from those of Wilson and Manery gives support to the idea that cytolysis of the cells in distilled water is sufficient treatment to remove all the analyzable K^+ and Na^+ from the

TABLE 2

K^+ and Na^+ content in the white blood cells of several vertebrates

ORGANISM	meq K^+/kg H_2O	meq Na^+/kg H_2O	% H_2O	REFERENCE
* Dogfish (Mustelus canis)	133.6 ± 10.6 (6)	88.6 ± 21.9 (3)	78.2	Hempling ('52)
Rabbit (polymorphs)	105.1 ± 6.1 (32)	67.5 ± 11.1 (21)	79.0 ± 1.14	
Rabbit (polymorphs)	106 ± 10 (18)	79.5 ± 7.8 (18)	79.25 ± 0.65	Wilson and Manery ('49)
* Horse	23	113	84.57	Endres and Herget ('29)

* Cells obtained from peripheral blood, by venipuncture.
( ) indicated number of samples.

cells. This then affords a more convenient and time-saving method for K^+ and Na^+ analysis than complete ashing.

It may be mentioned, in passing, that the term cytolysis as used here may be misleading, for the cells do not actually break up. If a suspension of cells in distilled water is examined under the microscope, it is found that the cells retain their spherical shape. The only marked difference from normal, other than absence of movement, is that the nucleus appears as a highly refractile body, with the lobes sharply outlined.

The values obtained for horse leukocytes differ markedly from those of the rabbit. In the horse, the content of Na^+ far exceeds that of K^+. Although, using the mammalian red blood cell as a comparable cell, it is not unusual to have species varying widely in their cation contents (Ponder, '48), yet, there is some possibility that these values for the horse leukocyte are not the normal values. The criticism rests upon the methods utilized in obtaining the horse cells. Briefly, the blood was obtained by venous puncture into oxalate as an anti-coagulant. The blood was allowed to settle for 24 hours at "lowest possible temperatures" and then the supernatant containing mostly leukocytes, platelets, and some red cells was removed. The leukocytes were then further separated from contaminating red cells by several centrifugations and washes with isotonic saline solution.

If the leukocytes of rabbits and horses are in any way comparable, there are several opportunities during the separation process for an exchange of cell K^+ for external Na^+. Oxalate as an anti-coagulant may exert the same harmful effects observed for citrate, thus rendering the cell permeable to cations. Exposing the cells for 24 hours to a low K^+, high Na^+ environment may involve a gradual replacement of cell K^+ by Na^+, as will be shown for rabbit leukocytes at 2°C. Finally, the several washings and resuspensions may produce the same degree of mechanical stress described previously (Hempling, '52), with the same type of accompanying K^+-Na^+ exchange.

The action of glucose on the K^+-Na^+ pattern. Preliminary studies had indicated that glucose was used up in the cell systems described. From several experiments, the average glucose concentration in the exudate fluids obtained by lavage with a saline or saline-buffer solution was 55 mg % initially. Experiments indicated that this supply of glucose was utilized in 6 to 12 hours at 37°C., with the usual concentration of cells obtained, namely 5,000 to 10,000/mm^3. Figure 3 shows the rate of glucose utilization under high and low glucose conditions.

Recently Manery et al. ('50) reported that leukocytes suspended in a very large volume of Ringer-Dale solution, with a K^+ concentration in the medium of 4.0 to 4.4 meq/l, had an internal K^+ concentration less than that of cells suspended in the same solution but with additional glucose present, after the two suspensions had been incubated at 37°C. for 90 minutes. In a personal communication ('52), she stated that the glucose-free Ringer-Dale suspension contained 3.0 mg % while the glucose suspension contained 160 mg %. On

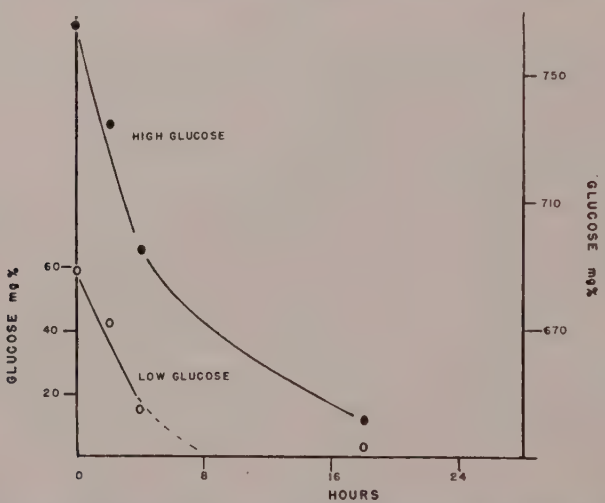

Fig. 3 Glucose utilization by rabbit polymorphonuclear leukocytes suspended in exudate fluid obtained by lavage with 0.95% saline solution.

the average, in 90 minutes, cells in the high glucose medium contained about 12 meq/l cell water more K^+ than cells in the low glucose medium. Could the diminished supply of glucose, then, contribute to the slow loss of K^+ observed over a 24-hour period?

The experimental procedure designed to determine an answer involved the use of exudates obtained by injection and lavage with saline-buffer solutions. The total exudates from three to six rabbits were pooled and aliquots were taken for the various systems to be tested. The several large ali-

quots in liter, glass-stoppered bottles, with an air space comprising 50–70% of the total volume were equilibrated at 37°C. by the roller technique from 45 to 90 minutes (Hempling, '52). The systems were then sampled for "0" time, after which

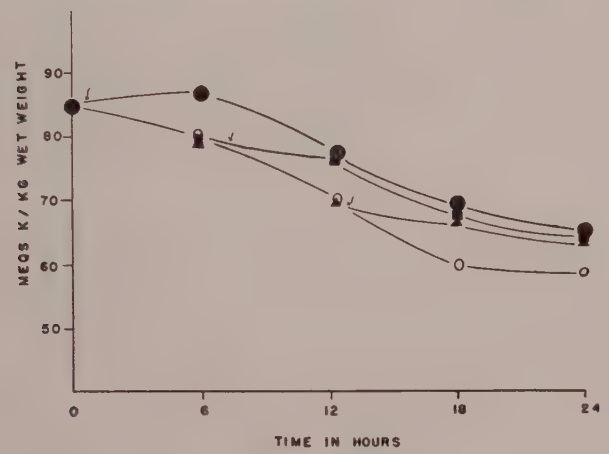

Fig. 4 The effect of adding glucose upon the rate of K^+ loss from rabbit polymorphonuclear leukocytes at 37°C., with external K = 2.6 meq/l and Na = 147 meq/l.

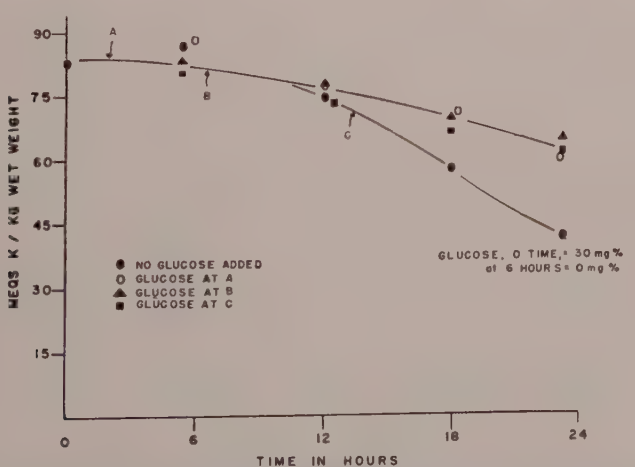

Fig. 5 The delayed effect of added glucose on the rate of K^+ loss from rabbit polymorphonuclear leukocytes at 37°C., with external K = 2.0 meq/l and Na = 149 meq/l.

glucose was added at designated times, depending on the type of experiment. Glucose was added in a saline solution to assure rapid mixing. Sterile conditions were observed throughout.

Results of these experiments are summarized in figures 4 and 5. In the presence of high concentrations of glucose, the total K^+ loss is always significantly less than that of cells in a low glucose suspension. The rate of K^+ loss may be slowed even after utilization of all the available glucose.

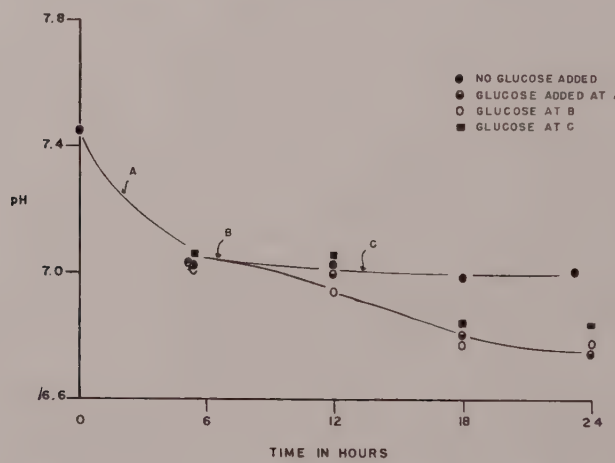

Fig. 6 Changes in pH accompanying the addition of glucose to exudate suspensions of rabbit polymorphonuclear leukocytes, at 37°C.

Glucose was added at intermittent periods during the experiment as indicated in the figures. The concentration of glucose after addition averaged 500 mg % in figure 4 and 800 mg % for figure 5. In figure 5, for example, after 13.5 hours, the rate of K^+ loss could be slowed by the addition of glucose, even though the system had been without glucose for at least 7.5 hours previously, since analyses had shown that by 6 hours, there was no initial glucose remaining.

The action of glucose is not fixed from experiment to experiment. At the end of 6 hours, in figure 4, there is a significant difference in the K^+ content of cells in high glucose

suspensions as compared to cells in low glucose cencentration. Yet in figure 5, a clear-cut effect of glucose is not evident until 18 hours, regardless of whether glucose had been added at two hours, 6.5 hours, or 13.5 hours.

Figure 6 shows the pH changes which occur in the environment during the K^+ changes in figure 5. The increased hydrogen ion production in the presence of added glucose is not sufficient to explain the retention of K^+. For these cells, one

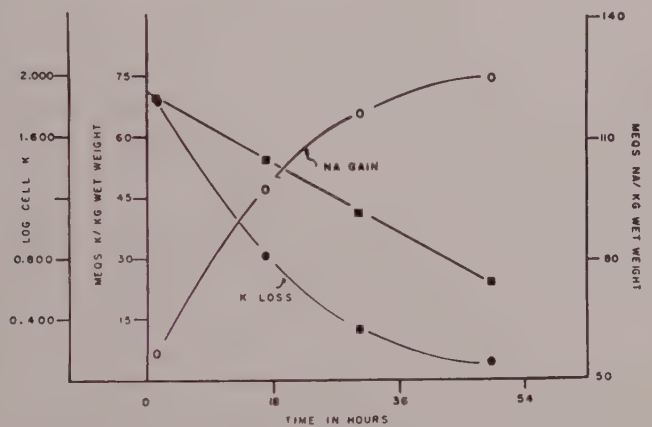

Fig. 7 The effect of low temperature on the rate of change of the K^+-Na^+ pattern in rabbit polymorphonuclear leukocytes. Temp. = 2°C.

cannot postulate a hydrogen ion exchanging for potassium ion, as has been done by Conway and O'Malley ('46) for yeast, since the loss of K^+ is accompanied by a reciprocal gain of Na^+ (Hempling, '53).

It would appear then, that the ability of glucose to retain K^+ in the cell requires a more complex relationship with the cell's metabolism than a simple ion exchange mechanism.

The effect of temperature. In view of the fact that metabolism appeared to be intimately associated with the leukocyte's ability to maintain its cation content, it became of interest to determine what effect a reduction in metabolic activity would have on the cation content. Lowering the temperature

to 2°C. was chosen as a means of reducing metabolism to a minimum.

Results of this type of experiment are shown in figure 7. The rate of K^+ loss is markedly enhanced when compared to the rate of loss of K^+ at 37°C. (cf. figs. 4 and 5). The uptake of Na^+ is exactly reciprocal to that of K^+ loss. In this particular experiment, the rate of loss may be expressed as a first order reaction with a $k = 0.0576\ hr^{-1}$. However, repeated experiments have shown that this is not always the case. A log plot will not always straighten out the curve, a fact which would indicate that more than a simple reaction was occurring at these low temperatures.

These losses of K^+ and gains of Na^+ at 2°C. were independent of glucose. The points on the curve represent samples from both high glucose (640 mg %) and low glucose (55 mg %) suspensions. The pH of the system remained constant at 7.4, indicating that no measurable glycolysis was taking place. Glucose analyses showed no utilization of glucose. When the leukocytes were examined at the end of the experiment, amoeboid motion was evident in all cells observed, after they were warmed to 37°C.

The results of a second type of temperature experiment are graphed in figure 8. Three aliquots of a pooled leukocyte suspension containing glucose were taken as experimental systems. Two were placed at 2°C. and the third at 36°C. They were all rolled at the same speed and sampled in duplicate at intermittent time intervals. At the time indicated on the graph, one system was transferred to 36°C. and sampled at two-hour intervals.

It may first be noted that there is a marked difference between the rates of K^+-Na^+ exchange at 2°C. and at 36°C. The K^+-Na^+ exchange at the higher temperature, from the shape of the curve, appears to be a complex function of several variables acting simultaneously. The rate of K^+ loss increases steadily over the first 24 hours, but then tapers off during the subsequent 24 hours. Na^+ exchange is not reciprocal during the early course of the experiment.

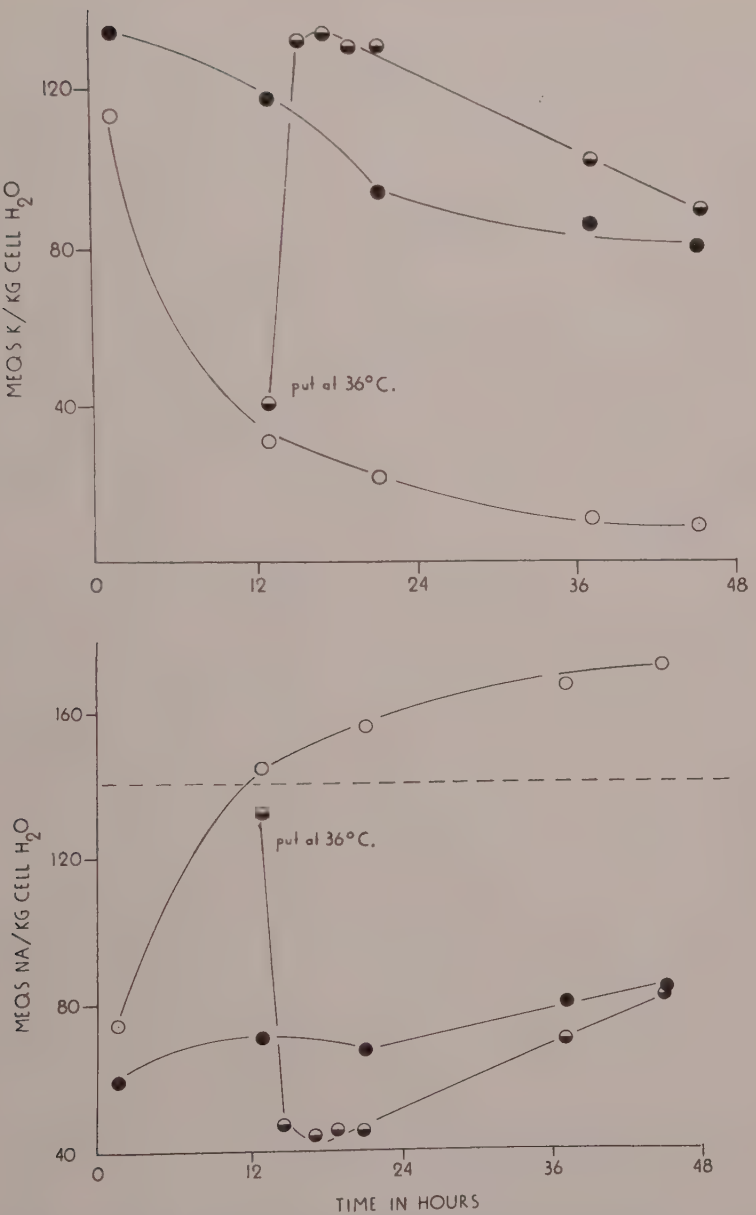

Fig. 8 Changes in the K^+-Na^+ pattern with shifts in temperature. External $K^+ = 3.3$ meqs/l, external $Na^+ = 140$ meqs/l. ○ = 2°C.; ● = 36°C.; ◒ = shift from 2°C. to 36°C.

At 2°C., the rate of K^+ loss is rapid initially, then slackens as the cell concentration approaches that of the environment (3.3 meqs/l). Na^+ gain is exactly reciprocal, within the experimental error. The dashed line marks the level of environmental Na^+.

Placing aliquot systems back to a 36°C. environment results in an accelerated accumulation of K^+ and a corresponding extrusion of Na^+, both against concentration gradients. Within two hours, approximately 100 meqs K^+/kg cell water have been transported from an environment of 3.3 meqs/l into a cellular compartment with a concentration of 42 meqs/kg cell water at the time of transfer to a 36°C. environment. An equivalent amount of Na^+ has been extruded from an initial cell concentration of 134 meqs/l into an environment of 140 meqs/l.

DISCUSSION

The leukocyte in suspension is a single cell system, with only one barrier between internal and external milieus. The ability of a cell to maintain a constant electrolyte pattern within its interior is a function of the effectiveness of this barrier. Previous work has shown that this barrier may be disrupted by the physical stresses of mechanical agitation (Hempling, '52). The work reported here serves to indicate that the stability of the barrier depends on the cell's metabolism as well.

Without metabolic activity, the K^+-Na^+ pattern cannot be maintained. This was strongly indicated by the ability of added glucose to slow the rate of loss of K^+ at 37°C. from cells in low glucose or glucose-free suspensions. But these types of experiments do not tell us whether the barrier to electrolyte movements is static or dynamic. One could as easily conceive of metabolism acting to keep the steric masonry intact as to maintaining a system of carriers shuttling back and forth.

It is in this connection that the experiments with temperature changes have proved so valuable. If the action of metabolism were such as to keep the barrier intact so that when

we lowered the metabolism, we allowed the electrolytes to seep through, restoring the metabolism could only stop the seepage; it could not restore what had been lost. The experiments reported above have shown that for the rabbit polymorphonuclear leukocyte such is not the case. Instead, what has been observed is a reaccumulation of the ion which has been lost and an extrusion of the ion which has entered the cell. Of necessity then, we must conceive of the barrier to electrolytes in this cell under normal conditions to be in some dynamic steady state.

If the metabolic machinery were geared for the extrusion of Na^+, and the K^+ movements were passive, then when the machinery was slowed down, as with a lowering of temperature to 2°C., one would expect the Na^+ to move in until its diffusion equilibrium was reached. Figure 8, however, indicated that by 12 hours, the cellular Na^+ had begun to exceed the external Na^+ concentration (140 meqs/l). In contrast, the equilibrium state for K^+ had still not been reached by 48 hours, the external K^+ concentration being 3.3 meqs/l.

Several alternatives could be supplied in considering the mechanism of K^+-Na^+ exchange at 2°C.

1. Complete loss of a carrier mechanism so that ions will move freely across a resisting boundary under their electrochemical gradients. This would imply that the rate of loss of potassium would exceed that of Na^+, since the concentration gradient for K^+ is higher (140 to 3, compared to 60 to 140). Under these conditions, the movement of Na^+ would be passive, with its apparent accumulation after 12 hours being attributed to its movement along an electrical gradient produced in the direction of the cell by the more rapid loss of K^+ out of the cell.

2. The loss, not of the carrier mechanism, but of its selectivity. In this case, the rate of exchange of Na^+ for K^+ at 2°C. would be determined either by the interaction of the carrier with the ions or the rate of movement of the complex through the barrier, or both (Solomon, '52). The rate of accumulation of K^+ and extrusion of sodium when the cells

are returned to 36°C. is approximately 50 meqs/kg cell water/hr. or approximately 10 times that observed in human red cells (Ponder, '50). There is reason to believe that this exchange rate is a minimum, since first analyses were taken at two hours, when the reaccumulation had been about completed (fig. 8).

Applying an hypothesis postulated for the red cell (Solomon, '52) to the polymorphonuclear leukocyte, it would be necessary to have an inward ion flux equal to or greater than 50 meqs/kg cell water/hr. when the cells were returned to 36°C., i.e., when metabolism would be working at a maximum, in order to permit accumulation of K^+ to occur. In this respect, some evidence by Wilson and Manery ('49) has shown a rapid exchange of Na^{24}. They obtained 90% exchangeability within 75 minutes. This is contrasted with a time requirement of approximately 10 hours for whole blood systems (Solomon, '52). Tullis and his associates ('53) have found a rapid potassium exchange which they claim is (cell for cell) approximately 1,000 times greater than red cells.

Obviously, one could increase the number of hypotheses by increasing the number of different types of action of metabolism. These few have been suggested because they can be tested for their validity, and therefore, suggest further experiments by which the leukocyte may be used in studies on electrolyte exchange.

SUMMARY

1. Analyses of rabbit polymorphonuclear leukocytes obtained from sterile peritoneal exudates gave values of 105 $\pm$ 6.1 meq/kg cell water for potassium, 67.5 $\pm$ 11.1 meq/kg cell water for sodium, and a per cent water of 79.0 $\pm$ 1.14.

2. Simple cytolysis of the cells in distilled water removed all analyzable K^+ and Na^+. Ashing was found to be unnecessary.

3. The addition of glucose was found to slow the rate of K^+ loss observed for cells suspended in exudate fluid at 37°C., regardless if the glucose were added initially or at intermittent intervals.

4. Studies on glucose utilization and hydrogen ion production were carried out in conjunction with electrolyte analyses. Protection against K^+ loss by glucose could not be attributed to simple hydrogen ion exchange for extracellular K.

5. Maintaining cell suspensions at 2°C. produced an enhanced loss of K^+ when compared to losses at 37°C. An exact reciprocal amount of Na^+ was gained.

6. Systems allowed to lose K^+ at 2°C., when then placed at 36°C., took up K^+ and extruded Na^+ in a reciprocal fashion against concentration gradients. Approximately 100 meqs K^+/kg cell water were transported in two hours from an environment of 3 meqs/l.

ACKNOWLEDGMENT

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SYNTHESIS OF RIBONUCLEIC ACID PURINES AND PROTEIN IN ENUCLEATED AND NUCLEATED SEA URCHIN EGGS

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The problem of the manner in which the nucleus of the living cell exerts its effects on the cytoplasm, and on the organism in general, is one of primary biological importance, since the nuclear apparatus, as it has been aptly described by Mazia ('52), can be considered as "the historical organ of the cell," whether considered "from a very long-range evolutionary view, a shorter genetic view, or the still narrower standpoint of ontogeny." The experiments in this paper were designed to give some insight into the nucleus-cytoplasmic relationship.

One method of attack upon this problem is to compare the metabolism of cells whose nuclei have been removed, with nucleated cells of the same type. In this manner it is possible to determine which metabolic reactions can be carried out in the cytoplasm of the cell, completely or partially independent of the influence of the nucleus.

There has been speculation that the ribonucleic acid (RNA) of the cytoplasm may have either a nuclear precursor (Marshak, '48), or that the RNA from the nucleus actually diffuses out into the cytoplasm (Jeener and Szafarz, '50). Part of the evidence offered to support these hypotheses are the well confirmed fact that nuclear RNA has a higher specific activity than the corresponding cytoplasmic nucleic acid, as determined by tracer studies in various tissues for short periods of time (Marshak, '48; Eliasson, Hammersten, Reichard and Aqvist,

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'51), and cytochemical observations that during nitrogen assimilation, the cytoplasmic RNA appears first in the area around the nucleus, followed by its spreading peripherally through the cell (Lagerstedt, '49). The following experiments attempt to show whether RNA can be synthesized in the cytoplasm of the sea urchin egg in the absence of the nucleus.

EXPERIMENTAL

Eggs were obtained from the sea urchin, *Strongylocentrotus purpuratus,* by injecting 1.0 ml of 0.5 M KCl into the body cavity. They were enucleated by a slight modification of the method of Harvey ('36). The eggs were overlayed on a solution made up by aiding 8 parts of 0.95 M sucrose to 2 parts of sea water. The mixture was centrifuged in a Spinco Model L Ultracentrifuge in 100 ml centrifuge tubes at 10,000 r.p.m. for 12 minutes. The enucleated fragments were collected at the bottom of the tubes and resuspended in 2.0 ml of sea water. Then they were overlayed on the sucrose solution again and recentrifuged as before. The second centrifugation yielded fragments which were 99% free of nucleated portions. The nucleated portions were obtained by combining the supernatant fluids, adding an equal volume of sea water to lower the specific gravity of the mixture, and centrifuging in the Servall centrifuge. The nucleated portions were contaminated by about 10% whole eggs, and 2% enucleated fragments, as determined by microscopic examination. For each incubation mixture it was necessary to pool fragments of each type in order to obtain enough material for isolation of the ribonucleic acid purines. The fragments were incubated with 2,000,000 counts of glycine-2-C^{14} (as measured in a gas flow counter) in 25 ml of sea water. Whole eggs were also incubated as controls. At the end of each incubation the reaction was terminated by addition of 8.0 ml of 25% trichloroacetic acid (TCA). The RNA purines were isolated by the method of Schmidt and Thannhauser as modified by Furst ('50). The purines were precipitated twice as the copper complex by the method of Hitchings ('41), plated on aluminum disks and counted in a

gas flow counter. The composition was kept constant by standardization of the copper precipitation procedure. The copper purine compound from each group of fragments was later pooled and the radioactivity found to be present in the adenine and guanine, which were crystallized as the picrate and sulfate respectively, recounted and the purity of the bases checked by determining the absorption curves in the Beckman spectrophotometer, or by running paper chromatographs (Markham and Smith, '51). The protein residue of the Schmidt Thannhauser method was washed twice with 5% TCA, twice with acetone, and then twice with ethyl ether after which it was plated on aluminum disks from suspensions containing 3 parts of petroleum ether to 1 part of ethyl ether. In some cases the protein was treated with performic acid in order to reduce the possibility of radioactivity residing in other than peptide bond linkages. The specific activity of the protein in these cases was not reduced.

RESULTS

The ability of the enucleated and nucleated fragments of the sea urchin eggs to synthesize RNA purines and proteins as measured by the uptake of glycine-2-C^{14} into these compounds is summarized in table 1. Although there is no net production of either RNA or protein, the incorporation of glycine into these two types of substances has been interpreted throughout this paper as being a measure of the synthetic mechanisms by which these larger molecules are built up from smaller precursors. Also the amount of RNA purine and protein from the various fragments was the same as calculated per unit of dry weight, and the assumption was therefore made that the precursor pools were of equal magnitude in the two types of fragments, and that the specific activities of the isolated purines and proteins were a measure of the amount of synthesis per unit time of the RNA purines and proteins, respectively. Both tables 1 and 2 show the results of independent experiments using different batches of eggs over a period of three months. In each experiment the enucleated fragment was able to syn-

TABLE 1

Comparison of the specific activities of RNA purines and of protein in enucleated, nucleated and whole sea urchin eggs

	EXPERIMENT 1			EXPERIMENT 2			EXPERIMENT 3		
	Incubation time	S.A. purines	S.A. proteins	Incubation time	S.A. purines	S.A. proteins	Incubation time	S.A. purines	S.A. proteins
	min.			*min.*			*min.*		
Enucleated egg	25	15	3.6						
	265	51	7.5	240	73	10	240	73	16
Nucleated egg	25	11	3.1						
	265	23	5.4	240	15	9	240	7.5	10
Whole egg	25	18	3.8						
	265	62	9.0	240	88	16	240	90	18

All incubations carried out with 2.0×10^6 counts glycine-2-C^{14} in 25 ml fresh sea water at 20–22°C.
Specific activity of purines expressed as cts./min./mg copper purines.
Specific activity of proteins expressed as cts./min./mg protein.

thesize more RNA purines than the nucleated fragment as shown in table 2. This ability varied from twice as much in experiment 1 to 10 times as much in experiment 3, when comparable incubation times were used. In the case of protein the differential was much less, the enucleated fragment synthesizing, at most, one and one-half times as much as the nucleated fragment. However, the whole egg was more active than either the enucleated or the nucleated fragment, probably illustrating that some damage to the cells occurred during the fragmentation and centrifugation.

TABLE 2

Ratios of specific activities

EXPERIMENT NO.	PURINES S.A. enucleated eggs / S.A. nucleated eggs	PROTEINS S.A. enucleated eggs / S.A. nucleated eggs
1[1]	2	1.4
2	5	1.1
3	10	1.6

[1] The values for experiment 1 represent those calculated from the longer incubation period.

Because of the small amount of material available for such experiments, no attempt was made to ascertain whether the synthetic mechanisms involved were confined to particular particles of the cytoplasm.

DISCUSSION

The results of these experiments are offered as evidence that, at least in the sea urchin egg, ribonucleic acid synthesis occurs in the cytoplasm in the absence of the nucleus or nucleolus. In this respect, the present work does not agree with the general hypothesis set forth by Marshak ('48) that cytoplasmic RNA has a nuclear precursor or with the idea of Jeener and Szatarz ('50) that nucleolar RNA diffuses through the nuclear membrane into the cytoplasm. The latter authors base much of their views on work with intact cells,

where over short periods of time the nuclear RNA achieves higher specific activities of radioactive tracers than the cytoplasmic RNA. However, the results of their experiments can, for the most part, be explained on the basis of high specific activity precursors for both types of RNA as suggested by Barnum and Huseby ('50).

These experiments do not, of course, rule out the possibility that nuclear precursors may contribute to a small degree to cytoplasmic RNA in sea urchin eggs, or to a major extent in other cells, but they do show that almost all of the synthesis in the sea urchin egg can be accounted for on the basis of cytoplasmic activity alone. In all the experiments performed here, the specific activity of the RNA purines in the enucleated fragment was higher than in the nucleated portion, which certainly would not be expected if the cytoplasmic RNA had a nuclear precursor. This is not surprising in light of the work of Shapiro ('35) and Ballentine ('40), who showed that enucleated fragments of Arbacia eggs had a greater oxidative capacity than the nucleated fragments.

Although the activity in the protein isolated for the enucleated cells was low, it was of the same order of magnitude as protein from the whole cells, and together with the finding that performic acid treatment of the protein does not reduce the specific activity, the author feels that it represents a low order of protein synthesis. Thus, this work illustrates that protein synthesis can occur in cells devoid of nuclei, and is in agreement with the experiments of Brachet ('51) on enucleated Acetabularia and with those of Peterson, Greenberg and Winnick ('50), on the incorporation of amino acids into cytoplasmic particles of mammalian cells.

The author is indebted to Dr. D. M. Greenberg for his interest, suggestions and encouragement during the period of this work. He also thanks Dr. Rudolf Stohler and Dr. Daniel Mazia for making available some of the sea urchins used in these experiments.

SUMMARY

Experiments with glycine-2-C^{14} are offered as evidence that ribonucleic acid purine and protein syntheses occurs in enucleated unfertilized eggs of the sea urchin, *Strongylocentrotus purpuratus*. The magnitude of incorporation into both the enucleated and nucleated fragments of the sea urchin eggs indicates that most, if not all, of the ribonucleic acid purines are synthesized in the cytoplasm in the absence of the nucleus.

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AZIDE AND THE EFFECT OF ACTIVITY IN FROG NERVE[1]

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THREE FIGURES

During a study of the effect of azide on the absolute refractory period of frog single nerve fibers (FitzHugh, '54), the effect of repetitive activity on the refractory period of whole nerve was tested, both with and without 0.2 mM azide. A half maximal compound action potential resulting from the test stimulus was used as a criterion of recovery. First, several repetitions (fig. 1) were made of an experiment originally reported by Field and Brücke ('26) and later repeated with better technique by Bugnard and Hill ('35) and Woronzow ('35). A noteworthy feature of these changes of refractory period is that their form closely parallels that of the changes of the rate of oxygen consumption (fig. 2). These two types of experiment were done under similar conditions except that phosphate buffer was used in measuring the refractory period, but bicarbonate buffer in the respirometer. Figure 3 shows one of two similar experiments on the effect of azide, in a concentration (0.2 mM) which eliminates the activity respiration, on refractory period changes such as those of figure 1. It is apparent that a nearly normal effect of activity on refractory period occurred even though the activity respiration was blocked. Too few experiments were done to determine

[1] This paper is based on part of a dissertation submitted to the Faculty of Philosophy of the Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy.

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whether the slight difference of shape between the curves of figures 1 and 3 was significant. However, it is obvious that the activity respiration is not essential for the recovery of the refractory period to its resting level after a long period of activity. Brink et al. ('52) found that the activity respiration was not necessary for conduction; its function in normal nerve, if any, therefore remains unknown.

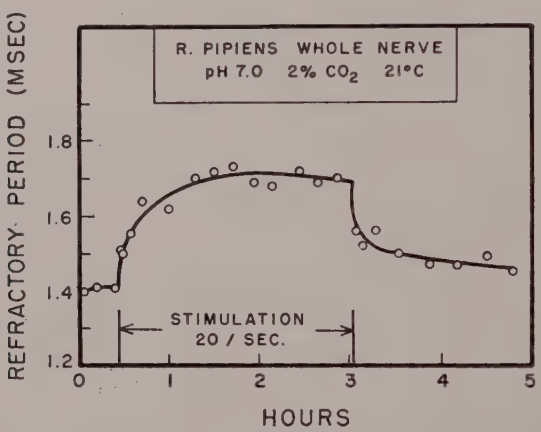

Fig. 1 Effect of repetitive activity on the absolute refractory period of whole nerve. Duration of refractory period is taken as the interval after the conditioning stimulus for a test stimulus of 5 times threshold intensity to produce a second A-alpha compound action potential of half maximum height.

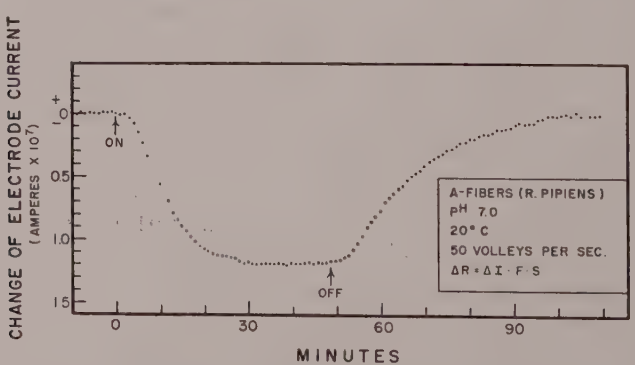

Fig. 2 Change in oxygen concentration at the oxygen cathode downstream from a nerve in a flow chamber, during and after a train of impulses. Oxygen concentration measured at one minute intervals. (Brink, Bronk, Carlson and Connelly, '52).

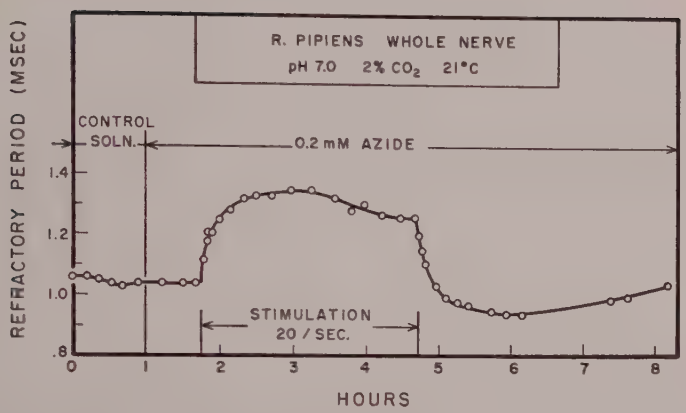

Fig. 3 Experiment showing that the transients of refractory period during repetitive activity (20/sec) are essentially normal, even though the activity respiration has been blocked by 0.2 mM azide. Whole nerve.

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EFFECTS OF AZIDE AND ELECTRICAL POLARIZATION ON REFRACTORY PERIOD IN FROG NERVE [1]

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THIRTEEN FIGURES

INTRODUCTION

Brink, Bronk, Carlson and Connelly ('52) have studied the release of chemical energy in frog nerve accompanying activity and in the presence of inhibitors, by measuring the rate of oxygen consumption with the oxygen-cathode respirometer. The object of the present study is to extend these observations by examining the changes of a functional, rather than a chemical, index of the metabolic state of an axon. Following a suggestion by Heinbecker ('29), the absolute refractory period was chosen for this purpose. Heinbecker found that anoxia caused a steady increase of refractory period of A-alpha fibers which progressed until the nerve became inexcitable after several hours without oxygen.

The total respiration of frog nerve consists of two components, the resting respiration and the extra, or activity, respiration which appears when the nerve conducts impulses. That this extra respiration is not necessary for the conduction of impulses is shown by the fact that 0.1 mM sodium azide selectively suppresses the extra respiration, leaving the resting respiration unchanged and without blocking conduction.

[1] This communication is based on a dissertation submitted to the Faculty of Philosophy of the Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy.

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In this paper evidence will be presented that the extra respiration is not immediately required for processes of recovery as measured by the absolute refractory period. However, a concentration of azide that depresses the resting rate somewhat causes a steady increase in refractory period eventually leading to inexcitability. Anodal polarization reverses this effect as long as excitability remains.

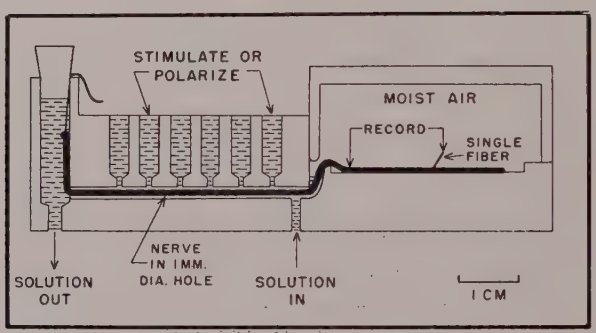

Fig. 1 Plexiglas flow chamber. The 6 stimulating-polarizing electrodes are columns of solution communicating with small calomel half-cells (not shown). Platinum wire recording electrodes.

METHODS AND APPARATUS

The nerve trunk of the Leopard Frog, *Rana pipiens,* that was used included spinal nerves VII–IX and the sciatic, peroneal, and tibial nerves, extending nearly to the ankle. The sciatic was stimulated, and for recording, a large A-alpha fiber was dissected from one of the distal branches for about a millimeter with sharp steel needles under a dissecting microscope.

Figure 1 shows the flow chamber, which was designed to duplicate the conditions imposed on the nerve in the flow respirometer of Carlson, Brink and Bronk ('50). The distal parts of the spinal nerves, and the sciatic, lay snugly in a 1 mm diameter hole in a plexiglas block. Bathing solution was drawn through the hole from a supply well at a rate of 4.25 mm^3/min. (2.55×10^{-4} liters/hour) by a motor-driven 1 ml tuberculin syringe. Six calomel cells communicated with

the 1 mm hole through 0.8 mm diameter holes spaced at 5 mm intervals. The distal branches of the nerve trunk passed into a moist chamber with a removable lid. The floor of the moist chamber was a few millimeters above the fluid level in the supply well, to prevent escape of the flowing solution into the chamber. The single fiber, after being dissected in the moist chamber, was placed on a recording electrode of platinum wire. The other recording electrode was a grounded platinized platinum wire touching the nerve trunk.

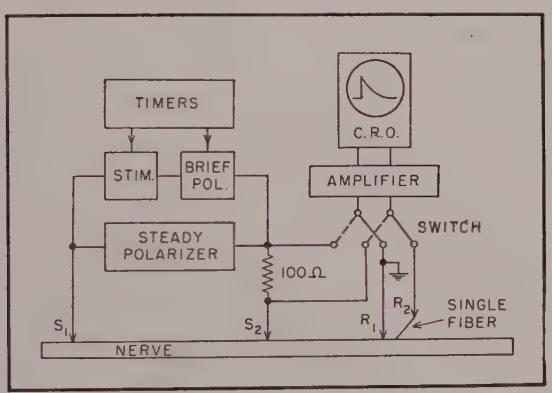

Fig. 2 Block diagram of circuits. Stimulator and brief polarizer act through radiofrequency links. Steady polarizer is a battery and voltage divider, connected through a one megohm series resistor and a hand switch. The A-C amplifier can be switched (left) to measure voltage drop across a 100 ohm resistor, or (right) to record single fiber action potentials.

The bathing solution was that used by Carlson, Brink and Bronk ('50), equilibrated with 50% oxygen, 2% carbon dioxide, and 48% nitrogen, and buffered with sodium bicarbonate to a pH of 6.9–7.0. Unbuffered solution was used in the moist chamber and for dissection. In making azide solutions, sodium chloride was partially replaced by an equivalent quantity of sodium azide.

Changing solutions took about a minute. After changing the solution in the supply well, $\frac{1}{2}$ ml of fresh solution was drawn past the nerve during a one minute period. In some experiments, large effects on refractory period appeared one

or two minutes after changing solution. Since the principle effects on refractory period studied were slower than this, any lag in changing solutions was negligible.

Figure 2 shows a block diagram of the stimulating and recording apparatus. Rectangular stimulating pulses 0.05 or 0.1 msec. long were delivered from a stimulator through radiofrequency (R-F) links (Schmitt and Dubbert, '49). The values of stimulus intensity reported were proportional to readings on the stimulator dial, which changed linearly with the amplitude of the pulse to within 5%. Brief rectangular polarizing pulses came from a second stimulator and R-F

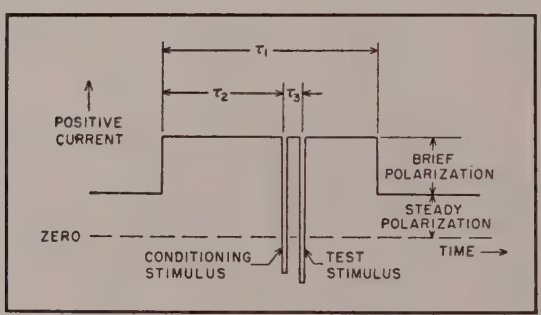

Fig. 3 Current form passed through nerve by the circuit of figure 2. Dial settings of stimulus intensity correspond to the amplitude of the stimulating pulse as measured from the baseline provided by the brief polarizing current.

link. Steady polarizing currents were delivered from a battery through a 1 megohm series resistor. This resistance was large compared to that of the R-F links and the nerve preparation, which were each about 20,000 ohms. Switching the steady polarizer on and off did not change the currents from the links by more than a few per cent. The rectangular pulses from the links were examined by connecting the A-C amplifier and oscilloscope across the 100 ohm resistor, in order to check that their amplitudes were not affected by the steady polarizer or by each other. Figure 3 shows the entire current form which could be applied to the nerve. This pattern was repeated usually at a frequency of twice a second.

Single-fiber action potentials were recorded with the A-C amplifier, displayed on the oscilloscope screen, and made audible with a loudspeaker. The recorded action potentials of single fibers were usually about 1 mV in amplitude, occasionally as much as 5 mV. The shape of the action potential varied greatly with the solution level and the dryness of the fiber, and its descending phase was usually much slower than that of a compound action potential (fig. 2). However, the shape was unimportant, since it was necessary only to detect its presence or absence. A single fiber gave detectable action potentials for 2 to 8 hours. When the spike height fell to the noise level, a millimeter or two more of the same fiber was dissected and lifted up on to the recording electrode.

All experiments were done with the flow chamber enclosed in a constant-temperature box at 20°C. except as noted.

The method of measuring refractory period will be described below.

RESULTS

A. Recovery curves

In the first experiments done, the compound A-alpha action potential was recorded from the whole nerve trunk. Instead of using, as a sign of recovery, the smallest detectable action potential evoked by the test stimulus — the usual criterion — a half maximal second action potential was used. The refractory period readings obtained by the latter method were more accurate and reproducible than those by the former methods, because the action potential height changed more rapidly with a given small change of stimulus interval when the action potential was half maximal than when it was barely detectable. The greatest possible accuracy was desired because the changes of refractory period caused by low concentrations of azide were small.

The recovery periods of the individual fibers in a nerve trunk are distributed over a range of values. By choosing a test stimulus interval for which the second action potential is half maximal, one divides the entire population of alpha

fibers into two groups, with recovery times greater and less than that interval. However, there is no proof that any given fiber will retain its relative position in this distribution of values during an entire experiment. To eliminate this complication, experiments with single fibers were undertaken.

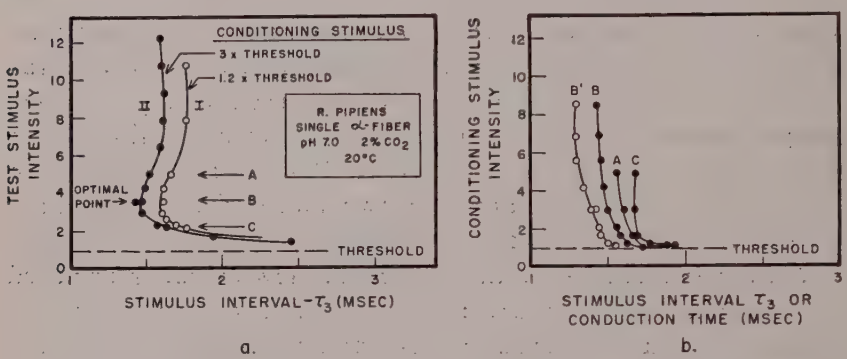

Fig. 4 a Recovery curves of single fiber for two different conditioning stimulus intensities. Abscissa is the greatest stimulus interval (τ_3 of figure 3) for which the second nerve impulse failed to appear. Ordinate is the test stimulus intensity expressed as multiple of threshold. In curve 1, conditioning stimulus is 1.2 times threshold; in curve II, 3 times threshold. Conditioning and test stimuli .05 msec. long, repeated at 2/sec., delivered through the same pair of electrodes 10 mm apart. Epineurium on.

b Effect of conditioning stimulus intensity on recovery interval of the fiber of a, for three different test stimulus intensities. Ordinate is conditioning stimulus intensity expressed as multiple of threshold. Abscissa for curves A, B, C is same as in a. These three curves were made with test stimulus intensities correspondingly marked in a. Abscissa for curve B′ is the total conduction time from stimulating cathode to recording electrode, using test stimulus intensity B. Conduction distance 35 mm.

It was found by Blair and Erlanger ('33) that if an excessively large test stimulus was used to measure recovery in frog single fibers, a larger value of recovery interval was obtained than if a somewhat smaller stimulus was used. Since they did not publish an illustration of the complete recovery curve, including the range of very strong test stimuli, such curves are given in figure 4 a, which shows the results of one of two similar experiments. The maximum interval between conditioning and test stimuli for which only one

response resulted from the pair of stimuli (the recovery interval) is plotted on the horizontal axis, and test stimulus intensity on the vertical axis. Such curves show one point, which we shall call the "optimal point," having a minimum recovery interval and occurring at a test stimulus intensity 3 to 4 times threshold. An increase of the conditioning stimulus intensity from 1.2 times threshold to 3 times threshold moves the whole curve to the left without changing its shape. In figure 4 b, curves A, B, and C show the effect of varying the conditioning stimulus intensity on the recovery interval of the same fiber. These three curves were made using three different conditioning stimulus intensities corresponding to levels A, B, and C of figure 4 a. The recovery intervals (abscissae) of curves A, B, and C decrease at the same rate as the conditioning stimulus is made stronger. Curve B′ of figure 4 b shows the effect of varying the conditioning stimulus intensity on the total conduction time from stimulating cathode to recording electrode, as measured from the beginning of the conditioning stimulus to the initial rise of the action potential. The test stimulus intensity was constant at value B. Assuming that the conduction velocity was constant, the decrease of conduction time with increasing stimulus intensity may have been due to either (1) a decrease of latency of the response at the stimulating electrode, or (2) a shift of the site of excitation toward the recording electrode by electrotonic spread. In either case, this observation can be explained without necessarily assuming that the recovery process, once started, is affected by the intensity of the conditioning stimulus, a conclusion in agreement with the all-or-none law. Thus the exact value chosen for the conditioning stimulus is not important. However, in the course of an experiment, the threshold sometimes varied, and to minimize errors from this source, it was decided to operate on that part of curve B (fig. 4 b) which is approximately parallel to the vertical axis. A conditioning stimulus intensity of three times threshold was therefore used in refractory period measurements.

The present results can be compared with those of Blair and Erlanger ('33), who found that on increasing the duration of the test stimulus the increase of recovery interval with increasing test stimulus intensity took place at a much higher multiple of threshold. In the experiment of figure 4 no change in the vertical position of this point was found when the test stimulus duration was increased from 0.05 to 1.0 msec. This difference may have been due to the difference in shape between the induction shock used by Blair and Erlanger and the accurately rectangular stimulus used in the

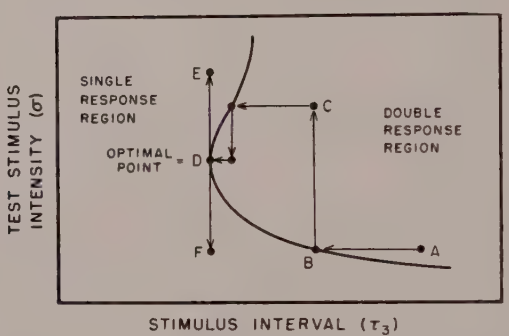

Fig. 5 Diagram showing typical sequence of adjustments of test stimulus intensity (σ) and stimulus interval (τ_3) made during a measurement of absolute refractory period of a single fiber. A to D: search for optimal point (D). DE and DF: final check that D is actually the optimal point.

present experiments. Unlike the latter, a negative inductorium shock is followed by a positive peak, which is relatively larger, the longer the duration of the shock.

In view of the above results, a standard procedure was adopted for measuring the absolute refractory period of single fibers, consisting of a sequence of adjustments of stimulus interval (τ_3) and test stimulus intensity (σ). Pairs of stimuli were applied at a repetition frequency of two per second. Typical adjustments are shown by the arrows of figure 5. When it appeared that point D had been reached, τ_3 was decreased just enough to eliminate the second response, and then σ was adjusted up and down (DE, DF) as a final

check. If the second response did not reappear, that value of τ_3 was taken as the absolute refractory period. The shape of the recovery curve and the nature of this final checking process made the single response, rather than the double one, the most convenient criterion of adjustment. One measurement could be made in 10–20 seconds. Readings were often reproducible to within .01 or .02 msec. with a steady preparation under control conditions. This accuracy was attainable only because the second response disappeared quite abruptly as τ_3 was decreased. A setting of τ_3 could usually be obtained for which the second response appeared randomly for some but not all of the test shocks, but a change of .01 msec in either direction usually was enough to produce a uniformly single or double response. The absolute refractory period of a fresh preparation at 20°C. was usually between 1.2 and 1.5 msec.

When the refractory period had been lengthened by azide or changed by polarization, the accuracy and reproducibility of the readings decreased considerably, either because the setting at the optimal point was less critical or because the refractory period was changing during the process of measurement. The variation of readings when they had been raised above 10 mesc. by 5.0 mM azide, for instance, was at least several tenths of a millisecond.

In the experiments to be described later in which polarization and azide changed the refractory period, complete recovery curves (as in fig. 4 a) were not obtained during the action of these agents. That the general properties of this curve remained the same is however shown by the fact that an optimal point was still usually found under these conditions. It was only when the refractory period reached very high values in axons treated with azide that this point is uncertain, since the state of the nerve was often changing so fast during the process of measurement that an adequate check of the existence of an optimal point could not be made. It should be mentioned that occasional alpha fibers were

found to have recovery curves without an optimal point, in control solution.

That the shape of the recovery curves of single fibers was not due to some peculiarity of the chamber is shown by the fact that recovery curves made with whole nerve trunks were of the classical shape (Erlanger and Gasser, '37) without an optimal point. This may have been because the optimal stimulus intensities for the individual fibers in a nerve trunk vary over such a wide range that any concavity to the right

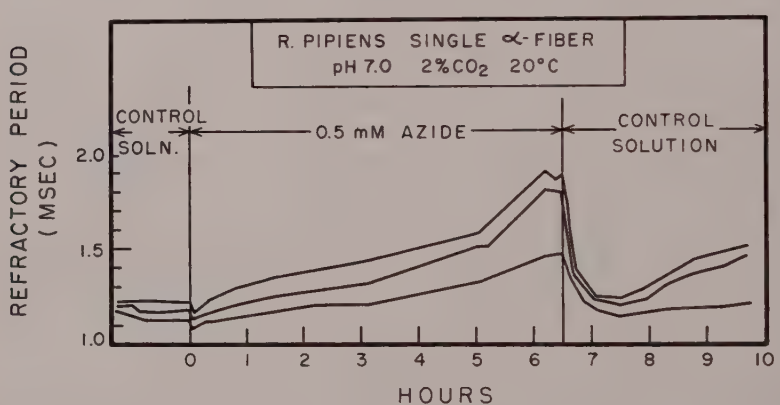

Fig. 6 Effect of 0.5 mM azide on refractory period measured at three different points along a single fiber. Epineurium had previously been removed and replaced.

is smoothed out and disappears from the compound recovery curve for a bundle of fibers.

B. Effect of azide on refractory period

Figure 6 shows typical changes of refractory period of a single fiber which was bathed in a 0.5 mM azide solution and tested at three different places along the nerve. The epineurium of this particular nerve had previously been removed and replaced for one of the experiments of section C, below. The transient decrease of refractory period during the first few minutes after changing solution was apparently not due to the azide, since a similar effect often appeared

even when the composition of the solution was not changed, but merely when a quantity of fresh solution was drawn past the nerve at a faster rate than usual.

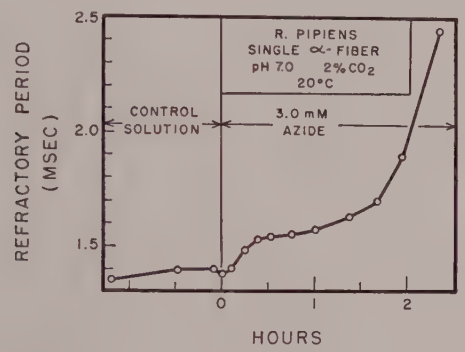

Fig. 7 Details of changes of refractory period of a single fiber upon application of 3.0 mM azide. Epineurium off.

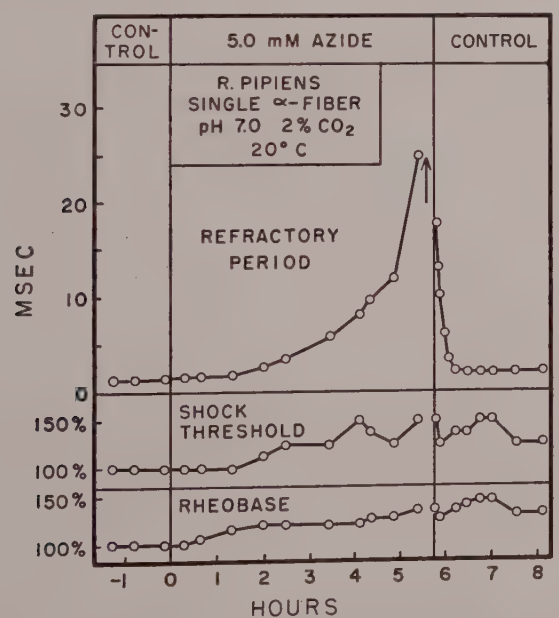

Fig. 8 Effect of 5.0 mM azide on refractory period, rheobase, and the threshold to a rectangular stimulus of 0.05 msec. duration. Vertical arrow indicates inexcitability. Sheath off.

Seven experiments on whole nerves and single fibers (with the epineurium in place) showed that a concentration of azide of 0.3 mM or less caused no increase of refractory period, at least within a period of an hour, while exposure to 0.45 mM or more caused a steady increase. One of these experiments has been published previously (fig. 17 of Brink et al., '52).

Higher concentrations of azide (3–5 mM) produced in general a similar effect to that of 0.5 mM, but with a higher rate of increase of refractory period. In addition, the initial part of the curve was more complex (see fig. 7). The epineurium was removed from the nerves in these experiments, to make them comparable to those described in section D, below.

Increases of refractory period of 20 times or more have been measured with only a moderate increase in threshold appearing until shortly before the onset of inexcitability. In one experiment (fig. 8), during 5½ hours in 5.0 mM azide, the refractory period increased from a control level of 1.44 msec. to 25 msec. During this time, the threshold to a .05 msec stimulus increased only 50%, the rheobase only 35%. Ten minutes later the fiber was inexcitable. After another 10 minutes, the control solution was applied; two minutes later excitability had returned. The refractory period decreased to 2.10 msec. after 40 minutes in control solution and remained nearly constant for the next 1½ hours. In another similar experiment, the refractory period reached 33 msec. after 5 hours in 5.0 mM azide, while the threshold to a .05 msec. stimulus increased only 50%.

Exactly how the threshold varied just at the onset of inexcitability could not be determined, since the condition of the fiber was changing rapidly as a result of the testing procedure. During this stage the fiber often responded to the first few stimuli at two per second, but not to later ones, making accurate measurements impossible.

C. Effect of the epineurium

A 0.5 mM solution of azide caused a slower increase of refractory period in desheathed nerves (epineurium removed)

than in normal nerves. Most of the curves of refractory period plotted against time were nearly linear for two or more hours after application of 0.5 mM azide (fig. 9). Figure 10 summarizes the results of 13 experiments of this type. Azide was less than half as effective after removal of the epineurium, while replacement of the epineurium gave results which were intermediate, but much more variable.

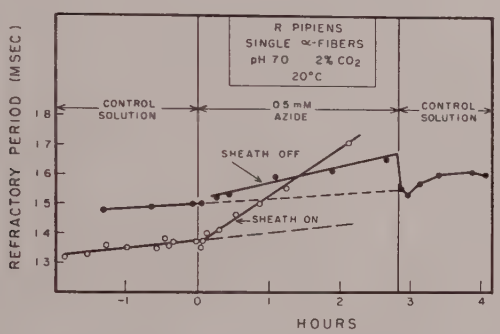

Fig. 9 Two typical experiments showing the effect of removing the sheath (epineurium) on the action of 0.5 mM azide on the absolute refractory period of single fibers.

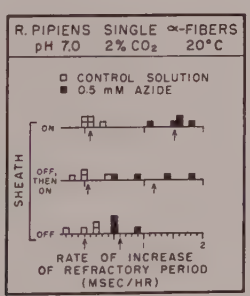

Fig. 10 Diagram showing the rate of increase of refractory period with 0.5 mM azide (black squares) and with control solution (white squares) for three different conditions of the sheath (epineurium). One black and one white square for each experiment.

Top row: Sheath not removed.
Middle row: Sheath removed and replaced.
Bottom row: Sheath removed.

In each row, the left arrow is the mean control value, the right arrow the mean value with azide.

If damage to the fibers resulting from removal of the epineurium were responsible for these results, fibers with epineurium removed and then replaced should have given data like those of desheathed nerve, or even more exaggerated in the same direction, but not intermediate. If the absence of the epineurium had increased the rate of diffusion of azide into the fibers, the effect of azide would have been greater in desheathed than in normal nerve, rather than less. It is possible that the epineurium hindered escape of some deleterious substance released in the fibers by azide.

All but three experiments were done in a flow chamber. The values of .11 and .15 msec./hr. (top row) and one value of .05 msec./hr. (bottom row) were obtained with the nerve in a moist chamber without flow and surrounded by a very thin film of solution, to reduce diffusion interchange. The three corresponding control values were .01, .00, and .02 msec./hr., respectively. In the moist chamber the effect of azide was therefore slightly less than in the flow chamber. If this difference is significant, it might be explained by a reduction of the available external azide supply, but not by a hindrance of the escape of a deleterious substance.

D. Effect of polarization

The fact that block of conduction by a number of different agents (anoxia, previous cathodal polarization, eserine, iodoacetamide, potassium, veratrine) can be relieved by anodal polarization has been interpreted by Lorente de Nó ('47) to mean that all of these agents act by decreasing the e.m.f. which maintains the membrane potential differences. Ionic current passing inward through the membrane is assumed to restore the membrane potential to the point where impulse propagation can occur. Whether or not this interpretation is correct, the action of a chemical agent such as azide may be analyzed through studying its interaction with the effects of electrical current through the axon membrane.

The epineurium was removed for all polarization experiments. Rashbass and Rushton ('49) conclude that the sheath,

because of its appreciable electrical resistance, markedly affects the current distribution of a nerve in contact with stimulating electrodes. Moreover, they found the sheath to be often not uniform, even in a branch-free region. Without investigating this question further, it was decided to do all polarization experiments on desheathed nerves. The sheath was cut completely around the nerve just distal to the sciatic plexus. The cut end of the sheath on the distal side of the cut

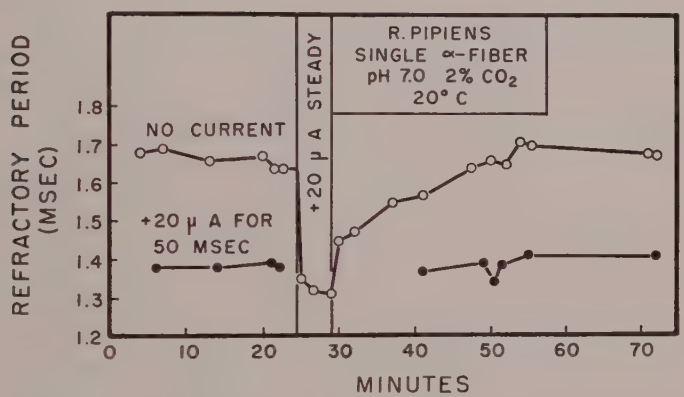

Fig. 11 Effects of steady and brief polarizing currents ($\tau_1 = 50$ msec., $\tau_2 = 30$ msec., repetition frequency 2 per sec.) on the absolute refractory period of a single fiber. Open circles: with 20 μA steady anodal or no polarizing current. Filled circles: with 20 μA brief anodal polarizing current. With steady current, refractory period underwent a rapid, then a slow change, and returned slowly to its original value after the steady current was switched off. With brief current, the rapid change appeared alone, and the return to a level characteristic of zero current, after current was switched off, was rapid. Epineurium off.

was then folded over on itself and gently peeled off inside out like a stocking.

The stimulating electrodes were used as polarizing electrodes. Polarization was first done in the usual way by applying a steady current, turned on and off by hand. It was soon found that the measurements were not adequately reproducible, due to the change of refractory period during polarization. Successive readings taken after turning on a steady current showed first a rapid change of refractory

period, appearing at the first reading, followed by a slower change in the same direction during the subsequent few minutes (fig. 11). When the current was switched off, similar rapid and slow changes occurred in the opposite direction. An interval of 20 or 30 minutes was sometimes necessary for the refractory period to return to the original control value. When, instead, a brief polarizing current was applied through an R-F link ($\tau_1 = 50$ msec., $\tau_2 = 30$ msec. in fig. 5), there was a reproducible change of refractory period of approximately the same size as the rapid change found with steady polarization, and this change was rapidly reversed after the current pulses were terminated. This method of polarizing was therefore chosen as a standard procedure.

The threshold of nerve is changed by polarization (Erlanger and Blair, '31). As a standard procedure, the conditioning stimulus was set at three times threshold (see section A) with zero polarizing current, but for currents which increased or decreased the threshold by a certain quantity, the conditioning stimulus intensity was increased or decreased by the same quantity, and was therefore no longer three times threshold. A value of refractory period measured during polarization depended somewhat on whether or not this arbitrary procedure was used, but any variations from this source were small compared to the effect of polarization itself.

Since the fiber was polarized and tested at a repetition frequency of two per second it was possible that the value of refractory period as thus measured reflected the action not only of the immediately preceding 30 msec. period of polarization, but also of the earlier ones. This point was not checked for all values of polarizing current, but it was found that during brief anodal polarization by a current 5 times rheobase, the refractory period readings were the same for repetition frequencies of 1, 2 and 5 per second. The effect of such polarization therefore appears within at most 30 msec. after switching on the current and is independent of any previous polarization more than $\frac{1}{5}$ sec. earlier. This test was not applied to azide-treated nerve, but it is possible that the re-

versal of the effect of azide by anodal polarization (see below) also occurred in as short a time as 30 msec. If so, this is much more rapid than the restoration of excitability by anodal polarization in an advanced stage of anoxia, which requires minutes (Lorente de Nó, '47, v. 2, Ch. XIII). The latter action resembles the slowly developing change in refractory period caused by prolonged polarization, as describer above.

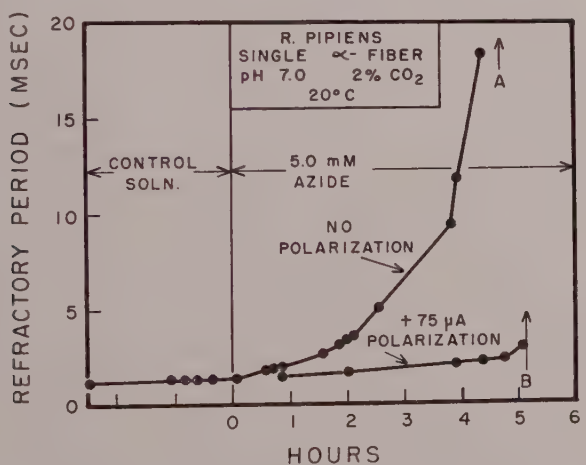

Fig. 12 Experiment showing the nearly complete reversal of the effect of 5.0 mM azide on the refractory period of a single fiber by anodal polarizing current (75 μA, 50 msec. duration, repeated twice a second, conditioning stimulus 30 msec. from beginning of polarization.) Arrows indicate when the fiber became inexcitable. Epineurium off.

Figure 12 shows an experiment in which the refractory period was measured with and without a brief anodal polarizing current of 75 μA, during treatment with 5.0 mM azide. In this particular fiber, rheobase was not measured, but in 4 others it was between 6 and 9 μA. The 75 μA current was thus about 10 times rheobase. The gradual lengthening of refractory period caused by azide during 4½ hours was almost completely reversed at any time by the pulses of anodal current.

In figure 12 even after inexcitability of the unpolarized fiber (arrow A), when the fiber failed to respond to some or all of the conditioning stimuli at 2/sec., the refractory period of the polarized fiber continued to be measurable for 20 min. This is reminiscent of Lorente de Nó's study of

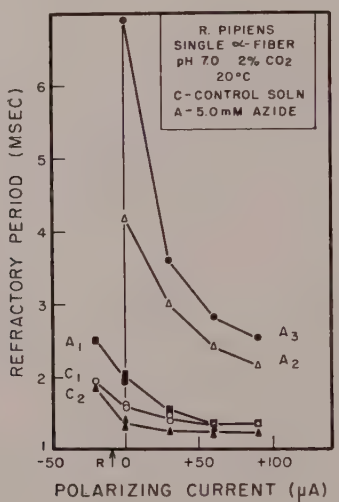

Fig. 13 An experiment similar to that of figure 12, but represented as curves of refractory period plotted against polarizing current. R = rheobase. Control curve C_1 was made 13 min. before application of azide. Later curves were made at three different times after azide was applied:

A_1 at 0 hr. 20 min.
A_2 at 2 hr. 45 min.
A_3 at 3 hr. 35 min.

Azide was replaced by control solution at 3 hr. 50 min., and control curve C_2 was made at 6 hr. 35 min. Epineurium off.

Thörner's phenomenon, in which inexcitability resulting from anoxia is relieved by anodal polarization.

Figure 13 shows a repetition of the experiment of figure 12. Since a number of values of polarizing current were used here, instead of just one, the results are presented as curves of refractory period plotted against polarizing current. Each curve represents a set of readings made within a period of about 5 min.; each of the times noted in the legend of figure

13 corresponds to the middle of such a period. The first and final control curves, labelled C_1 and C_2, have typical shapes (Bishop and Erlanger, '26, give similar curves obtained with steady polarization). The effect of azide is to increase the refractory period steadily for all values of polarizing currents, but this increase is less, the greater the anodal current.

The approximate constancy of threshold shown in figure 8 did not appear in these experiments; the combined effects of azide and of polarization caused a marked increase in threshold.

DISCUSSION

Rosenblueth, Alanís and Mandoki ('49), studying the recovery process in cat nerve, concluded that the absolute refractory period (defined by them as the stimulus interval giving the first detectable compound action potential in response to the test stimulus) probably has no physiological meaning, but that the significant period is their "functional refractory period," defined as the "time interval after a conditioning impulse within which the nerve cannot propagate another impulse." They continue: "The nerve can be stimulated before the expiration of this interval, however, and can respond to this stimulation, but this response will propagate only after the functional refractory period has elapsed." The functional refractory period is therefore longer than the absolute refractory period. According to this point of view, the absolute refractory period depends on the ability of the fiber to retain the effects of an applied stimulus, as a local response, until such time as recovery of excitability takes place (Rosenblueth and Luco, '50). However, granting that the functional refractory period marks an event of greater physiological significance than the absolute refractory period, the absolute refractory period remains a valid though nonspecific measure of the initial stages of the processes of recovery, and its use seemed more pertinent to the present study.

The changes of threshold and refractory period during treatment with 5.0 mM azide can be contrasted with those

found by Heinbecker ('29) during anoxia. With azide, the threshold stimulus strength remained relatively constant, increasing by only 50%, while the refractory period increased 20 to 30 times. In one experiment of Heinbecker, on the other hand, after 130 min. of anoxia, the threshold and absolute refractory period had increased together by nearly proportional amounts, reaching values of 3.7 and 3.4 times their control values, respectively. Unlike anoxia, azide affected the threshold much less than it did the refractory period. Five millimolar azide inhibits about 80% of the resting respiration, and the remaining 20% may be sufficient to maintain excitability, but not a normal rate of recovery. This result suggests that inexcitability resulting from azide may be of a different kind than that usually obtained from the action of narcotics, and may result from an inability to pass from a state of excitation to a state of rest, rather than the reverse. It may be that there is some substance which is used up during excitation and fails to be replenished in the presence of azide, and that when the concentration of this substance falls below some critical level, the threshold suddenly rises rapidly and inexcitability results.

Lorente de Nó ('47) has suggested that the relief of conduction block by anodal polarization indicates that the block is caused by a loss of membrane potential. In the present experiments, the reversal by anodal polarization of the effect of azide on refractory period does not necessarily mean that azide acts by depolarizing the membrane. Even in a nerve in control solution, the effect of anodal polarization is to decrease the refractory period, an effect which may or may not result from an increase in membrane potential. Although anodal polarization antagonizes the effect of azide on the refractory period, it may act in an entirely different way. Keeping this reservation in mind, one may assume as a simple working hypothesis that azide displaces the state of the nerve gradually to the left along the control curve of figure 13, while anodal current moves it back toward the right. This assumption is nearly satisfied by the curves of figure 13,

although too small a range of values of current was used to test it adequately. These effects may be assumed to result from the actions of current and of azide on the membrane potential, which in turn governs the rate of recovery following excitation.

This hypothesis appears reasonable in view of one of the known biochemical effects of azide, namely its ability to uncouple the synthesis of adenosine triphosphate from cellular oxidation reactions (Loomis and Lipmann, '49), an effect which may be important in nerve (Brink et al., '52). Since the maintenance of the e.m.f. of the nerve membrane requires a continuous supply of chemical energy, and since adenosine triphosphate plays an essential role as a bearer of such energy in many biochemical systems, it is possible that the following sequence of events occurs: azide inhibits the formation of adenosine triphosphate (which is therefore slowly exhausted), the membrane potential decreases, and the refractory period steadily increases. Then, when an anodal polarizing current is applied, it restores the membrane potential, and the refractory period decreases again.

Hodgkin and Huxley ('52) have recently given an explanation of refractoriness based on their theory of the squid giant axon: "According to our theory, there are two changes resulting from the depolarization during a spike which make the membrane unable to respond to another stimulus until a certain time has elapsed. These are 'inactivation', which reduces the level to which the sodium conductance can be raised by a depolarization, and the delayed rise in potassium conductance, which tends to hold the membrane potential near to the equilibrium value for potassium ions." (See their fig. 19.) Their figure 20 shows (for the non-propagated or membrane action potential) an absolute refractory period lasting about twice the duration of the spike and ending during the positive after-potential. Assuming for the moment that a similar explanation is valid for frog nerve, one can speculate on the possible ways that azide might act. It is not evident, however, just how the coefficients and functions

appearing in Hodgkin and Huxley's differential equations would have to be changed by azide to increase the refractory period without greatly changing the threshold. If azide merely slowed the return of h (The inactivation factor for sodium conductance) from its minimum value during the action potential to its resting value, the refractory period would be lengthened, but the action potential would be unchanged. However, if azide slowed the return of g_K (the potassium conductance) from its maximum value during the action potential, then the refractory period would be lengthened, the positive after-potential would be lengthened and perhaps increased in magnitude, and the total membrane conductance would return its resting level more slowly than in control solution. These last two effects could be detected in action potential records and impedance measurements, respectively.

The mechanisms of action of agents such as azide and applied membrane current on recovery processes in frog nerve still seem to be quite inaccessible to analysis. On the other hand, the Hodgkin-Huxley theory analyses the mechanisms of the squid axon membrane into several different processes, each experimentally measurable. If experiments on refractory period like those in the present paper could be combined with a theory like that of Hodgkin and Huxley on the same nerve preparation, it would perhaps be possible to settle the question of whether azide and applied current act in similar or in different ways.

ACKNOWLEDGMENTS

The problems examined in this experimental investigation arose from an extensive study of the relations between oxidative metabolism and the electrochemical properties of nerve cells, carried on by Dr. D. W. Bronk, Dr. F. Brink, and Dr. C. M. Connelly of the Biophysics Department of the Johns Hopkins University. The writer wishes to thank Doctor Brink especially for suggesting the problem, for his constant guidance throughout the research, and for his advice in preparing

the manuscript, and Mr. J. P. Hervey for advice on the electronic equipment. The experimental work was supported in part by a grant to Doctor Bronk from the Supreme Council, Scottish Rite Masons.

SUMMARY

1. The recovery curves of single A-alpha fibers of the frog showed a minimum recovery interval for a test stimulus of intensity 3 to 4 times threshold. The absolute refractory period is defined as this minimum recovery interval.

2. Azide in concentration of 0.5 mM caused a steady increase of refractory period of about 0.15 msec./hr. (in nerve with epineurium) for several hours.

3. The removal of the epineurium decreased the effect of 0.5 mM azide by more than one-half.

4. In fibers treated with 5.0 mM azide, the refractory period increased by a factor of 30 or more during 5 hours, while the threshold increased only 50%. Inexcitability occurred shortly thereafter.

5. Until inexcitability occurred, an anodal polarizing current about 10 times rheobase eliminated most of the increase of refractory period caused by 5.0 mM azide.

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RESPONSES OF A MOLLUSCAN SMOOTH MUSCLE TO ACETYLCHOLINE AND 5-HYDROXYTRYPTAMINE[1,2]

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FIVE FIGURES

INTRODUCTION

The simple, parallel arrangement of the long, unstriated muscle fibers of the anterior byssus retractor muscle of *Mytilus edulis,* L. makes possible certain mechanical and electrical investigations which would be impossible or very difficult with the more complex vertebrate smooth muscles. Just as the long-fibered frog sartorius has long been a useful object in the study of the properties of striated muscle, the byssus retractor can serve to clarify many problems in smooth muscle physiology. It has been employed by Winton ('37), Fletcher ('37 a, b, c), Van Nieuwenhoven ('46), Prosser, Curtis and Travis ('52) and Johnson and Perkins ('52), in detailed studies of mechanical and electrical responses to electrical stimulation. Singh ('37, '38a, b, c, '43) has investigated the effects of a wide variety of ions and drugs on this muscle, but his results do not establish the physiological significance of the responses to pharmacological agents.

[1] The major portion of this research was conducted while the author was an Atomic Energy Commission Predoctoral Fellow in the Biological Sciences of the National Research Council.

[2] This work was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service to Prof. J. H. Welsh at the Biological Laboratories, Harvard University.

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Because of the anatomical specialization of this muscle, measurements of changes in demarkation potential accurately reflect changes in membrane polarization. Correlation of mechanical responses to pharmacological agents with alterations in membrane polarization has been used in this study to cast further light on the duality of the contractile responses of this muscle. This duality was first studied by Winton ('37) and has been of interest to all subsequent investigators.

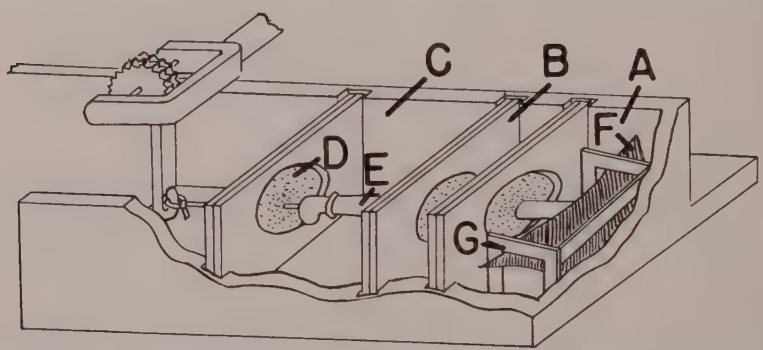

Fig. 1a The muscle chamber: (A) Compartment to be filled with KCl. (B) Compartment to be filled with oil. (C) Sea water compartment. (D) Rubber diaphragm in celluloid partition. (E) Byssus retractor muscle. (F) Shell triangle. (G) Celluloid frame to hold shell.

Another major aim of this study has been to clarify the physiological significance of acetylcholine in excitation, and of certain other agents in inhibition of the byssus retractor muscle.

METHODS

Correlation of the mechanical and electrical responses to pharmacological agents

Apparatus (figs. 1a and 1b). The muscle lay horizontally in a lucite chamber which was divided into three compartments by removable rubber-celluloid partitions. The chamber was mounted on a microscope ratchet and could be moved in a horizontal plane to adjust for changes in muscle length during equilibration.

A steel wire, bent at right angles and pivoted as a heart lever, was attached at one end to a thread from the muscle, and at the other to a wire pulling on an isotonic-isometric lever system similar to that described by Fletcher ('37c). A 20 gm weight kept the muscle stretched with constant force. This system permitted only slight shortening, ordinarily about 1.0 mm or 2.5% of the muscle's total length.

A Millivac millivoltmeter was used to record demarkation potentials. Potential differences were read at intervals of 15 seconds or longer. The electrodes consisted of electrolytically-

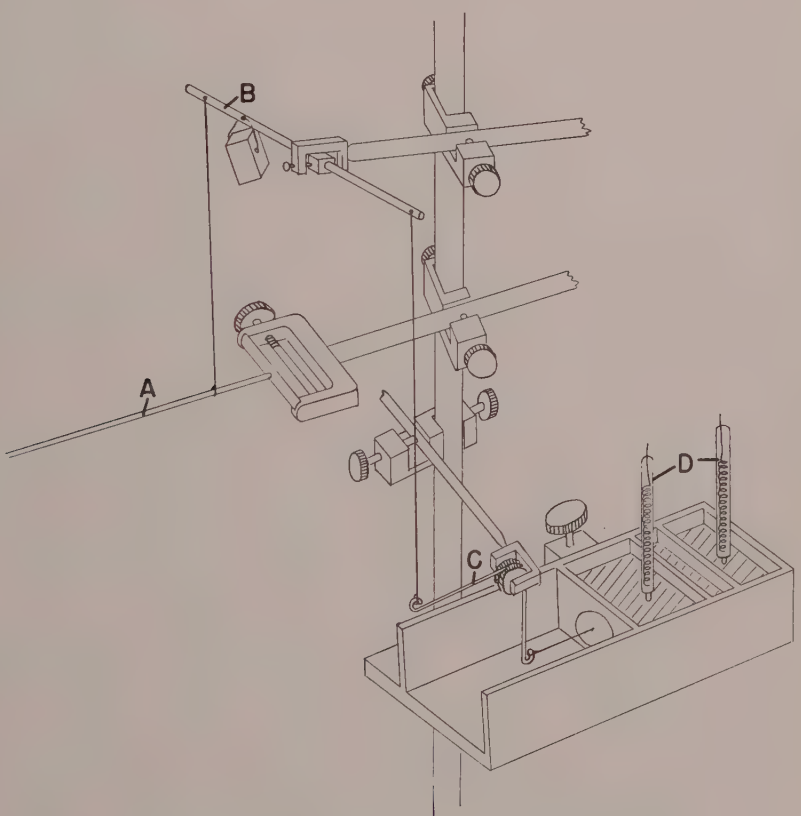

Fig. 1b The lever system: (A) Isometric lever. (B) Isotonic lever. (C) Right angle lever. (D) Electrodes.

chlorided coils of silver wire, sealed into lucite tubes filled with sea water. Wooden pegs made contact with the muscle.

Experimental procedure. Mussels (*Mytilus edulis,* L.) were collected at Nahant, Massachusetts or shipped from the Marine Biological Laboratory at Woods Hole. These were maintained for weeks in excellent condition in tanks of shallow sea water at 6°C.

A small triangle of shell around the insertion of the anterior retractor byssus muscle (ARBM) was cut out with a hacksaw or emery wheel. The muscle was opened by cutting the anterior and posterior adductor muscles along the midline. The ARBM was stripped of connective and nervous tissue and freed at the byssal end by a cut through the byssus organ posterior to the origin of the muscle. A thread tied about the byssus ran to the lever system. The shell triangle was held in the muscle chamber. This method of dissection and of mounting the preparation permitted recordings from muscles which were intact at both ends, a factor of importance since the individual fibers run the length of the muscle. This system eliminated the extreme tonic contraction other investigators have noted during dissection. It is probable that tonic contraction during dissection is always the result of mechanical stimulation.

The muscle was equilibrated in sea water at a temperature between 14.5° C. and 16°C. until it had been stretched to a constant length under the influence of the weight.[4] First the sea water in the narrow central compartment was replaced by mineral oil, which prevented leakage or diffusion of drug solutions between the two end compartments. Isotonic KCl was then added to the compartment holding the shell end of the muscle; the byssal end remained in sea water. The experiments began when the potential difference between the segment of muscle in KCl and that in sea water reached a

[4] It is very probable that this constant length represents a true "resting" length. A fully-equilibrated muscle will never stretch beyond this point although stimulated electrically or by pharmacological agents. The responses to a given concentration of acetylcholine are very uniform after equilibration.

steady level (usually after 30 minutes). The sea water chamber was drained and drug solutions poured in. Continuous recordings of mechanical events were made on a smoked drum while the demarkation potentials were plotted. All readings were made with reference to the depolarized segment.

Solutions of pharmacological agents were made up in molar concentrations (with reference to the active base).[5] Only fresh solutions of pure crystalline agents were applied.

Determination of the cholinergic nature of excitation in the ARBM

Bioassay for acetylcholine. Muscles were dissected free of ganglia and nerve trunks, rapidly weighed and ground with sand in a known minimal quantity of eserinized sea water at pH 3. This suspension was heated in boiling water for 5 minutes, cooled for 30 minutes and centrifuged. The supernatant was drawn off, neutralized, and assayed on the Venus heart (Welsh and Taub. '48). As a control, other muscles were ground in uneserinized sea water and assayed after standing one hour at room temperature.

Cholinesterase determination. A modification of the standard manometric technique (Stedman and Stedman, '35) was employed. Bicarbonate Ringer consisted of 5 ml of 1.26% $NaHCO_3$ freshly added to 25 ml of sea water. Muscles were ground with sand in sea water and the homogenate centrifuged. An aliquot of the supernatant was drawn off for assay. After equilibration (between 20°C. and 23°C.) for 10 minutes, the flasks were gassed with a mixture of 95% N_2 and 5% CO_2; 5 minutes later, the contents of the side arm were tipped in, and following an additional 5 minute equilibration, readings were made at 10 minute intervals for an hour. Non-enzymatic

[5] Acknowledgements are due to the following pharmaceutical firms, who kindly supplied the drugs used in these experiments.

Sterling-Winthrop (l-epinephrine bitartrate; Mytolon).

Searle (Banthine).

E. R. Squibb (d-tubocurarine chloride).

Abbott (Serotonin creatinine sulfate).

hydrolysis was automatically corrected as in the work of Zacks and Welsh ('51). Substrates were acetylcholine bromide, acetyl-β-methylcholine chloride and benzoylcholine chloride.

Determination of the nature of inhibition in the ARBM

Qualitative bioassay for epinephrine. Muscles were ground with sand in acidified frog Ringer's. A small quantity of serum cholinesterase was added to the homogenate to eliminate the necessity of atropinizing the heart. The homogenate was then centrifuged for 15 minutes. The supernatant was assayed on a Straub frog heart preparation.

Assay for 5-hydroxytryptamine, (serotonin; enteramine). Acetone extracts were made according to the method of Erspamer ('48). Aliquots of extract were dried at reduced pressure, rediluted in distilled water, and assayed on the Venus heart, the ARBM and the isolated atropinized uterus of an estrous rat. The Venus heart was soaked for at least 10 minutes in 10^{-5}M Mytolon before assaying the mixed extracts. The acetylcholine block produced by this method is complete, and is not reversed when the Mytolon solution is washed off. The activity of the extract was matched by known doses of 5-hydroxytryptamine (serotonin creatinine sulfate) and from this the 5-hydroxytryptamine content of the extract was calculated.

RESULTS

Correlation of the mechanical and electrical responses to pharmacological agents

Isotonic KCl (fig. 2). After a minute in isotonic KCl, the potential difference between the treated and untreated segments reaches 4 or 5 mv. The segment in KCl is negative. Following this initial rapid depolarization, the treated segment continuously depolarizes at a slower rate. The rate of depolarization steadily falls off and a constant potential difference is reached within 30 minutes or an hour. This po-

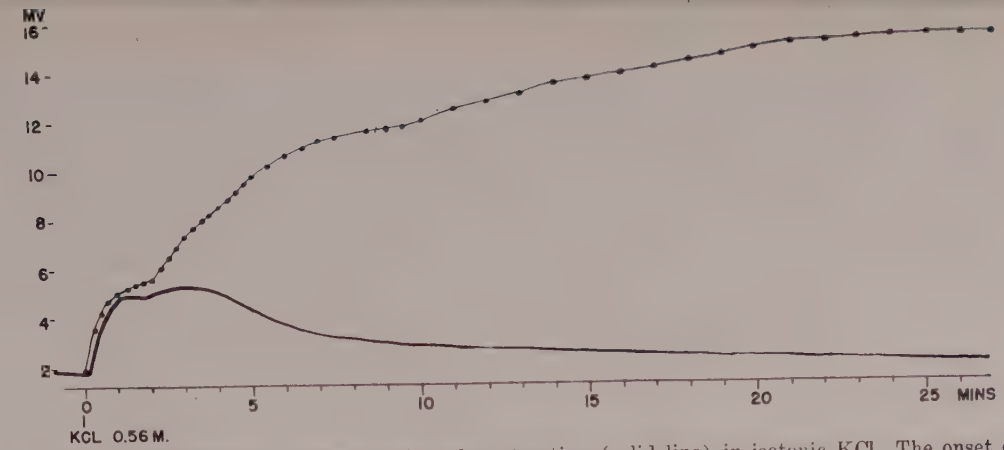

Fig. 2 The course of depolarization (dotted line) and contraction (solid line) in isotonic KCl. The onset of depolarization and contraction are arbitrarily superimposed at zero time in this and in all records to follow.

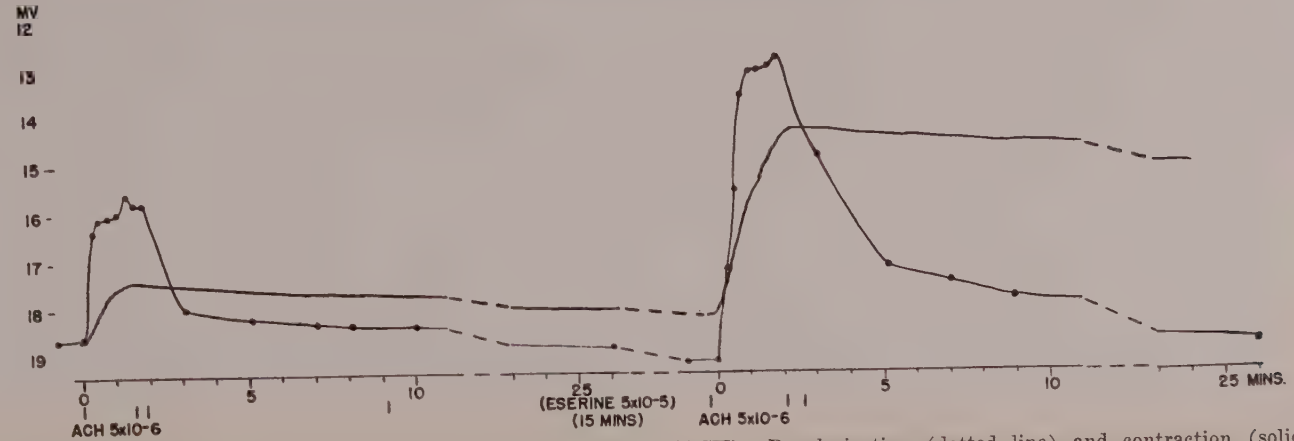

Fig. 3 Excitation: the response to 5×10^{-6} M. acetylcholine (ACH). Depolarization (dotted line) and contraction (solid line) are both potentiated by eserinization. Note the change in the course of repolarization after eserine. Double vertical lines indicate washing.

tential difference amounts to between 18 and 24 mv. The contractile response associated with the depolarization is distinctly diphasic: there is a brief period of rapidly rising tension, followed by a longer interval during which this tension level is partially maintained. Within 10 to 20 minutes, relaxation has been completed, although the muscle continues to depolarize steadily.

The KCl effect has not been studied in detail. It is noteworthy, however, that the course of depolarization in this invertebrate smooth muscle parallels in most respects the KCl depolarization observed in the long-fibered frog sartorius by Sandow and Mandel ('51).

Excitation: the acetylcholine response (fig. 3). Acetylcholine characteristically produces depolarization and contraction when applied in concentrations of 10^{-6} M. or higher (as high as 10^{-2} M.). The extent of the depolarization and the height of contraction increase with the acetylcholine concentration. The maximum depolarization and contraction are reached within a few minutes of treatment; gradual repolarization and relaxation ensue on prolonged soaking. When the acetylcholine solution is removed as soon as depolarization and contraction are maximal, then the muscle almost immediately repolarizes, while the contraction very gradually relaxes. In some cases there is a rapid partial relaxation after washing followed by a gradual relaxation to the original baseline, but typically the contraction is entirely tonic in nature.

Soaking the muscle in 10^{-5} M. eserine for longer than 15 minutes lowers the acetylcholine threshold several fold, to between 10^{-7} M. and 10^{-6} M. There is marked potentiation of acetylcholine depolarization and of contraction. Gradual repolarization during prolonged soaking in acetylcholine is totally abolished and repolarization after washing is no longer immediate, but follows an exponential course.

Some acetylcholine blocking agents have preliminary acetylcholine-like or acetylcholine-potentiating action. Of the blocking agents which by themselves produce only simple block,

atropine is among the least effective, while d-tubocurarine is more powerful and Banthine (β-diethylaminoethylxanthine-9-carboxylate methobromide) an autonomic blocking agent, (Hambourger, Cook, Winbury and Freese, '50) the most effective. As the concentration of these agents is increased, the extent of block increases. To the extent that depolarization by acetylcholine is blocked, the accompanying contraction is reduced. However, after the acetylcholine has been washed off, and the phase of rapid repolarization is complete, these blocking agents do not affect, in any concentration, the course of relaxation.

Relaxation: the responses to epinephrine and to 5-hydroxytryptamine (serotonin, enteramine) (figs. 4 and 5). Epinephrine applied to a fresh muscle in a concentration between 10^{-8} M. and 10^{-3} M. causes depolarization and contraction; it summates with applied acetylcholine. In a few muscles this effect is not seen. In all muscles, repeated brief application of low concentrations of epinephrine, or soaking for 10 minutes in concentrations of 10^{-4} M. or higher, reverses the action of epinephrine. At this point, if the muscle is in a state of tonic contraction, due, for instance, to previous treatment with acetylcholine or incomplete equilibration, then sudden relaxation ensues, without any detectable change in demarkation potential. Following epinephrine reversal, responses to subsequent doses of acetylcholine are strikingly altered. The initial depolarization and contraction produced by a given concentration of acetylcholine are both potentiated. The greatly potentiated contraction, however, relaxes immediately upon washing. There is no alteration in the usual rapid course of repolarization. Maintaining a high concentration (10^{-4} M.) of epinephrine in the bath entirely eliminates the prolonged tonic phase of the acetylcholine contraction, while the initial effects of applied acetylcholine are not blocked, but potentiated.

The response of the muscle to 5-hydroxytryptamine is more striking than the epinephrine response. Neither depolarization nor contraction is seen when concentrations ranging from

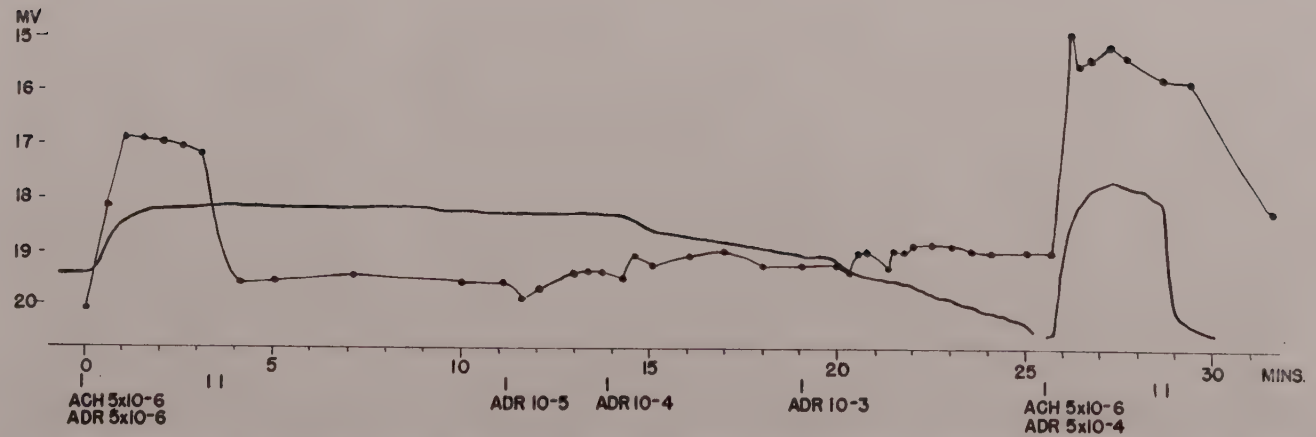

Fig. 4 Relaxation: the interaction of epinephrine (ADR, in molar concentrations as indicated) and acetylcholine (ACH) 5×10^{-6} M. The contraction (solid line) produced by mixed acetylcholine and epinephrine is relaxed by high concentrations of epinephrine. After relaxation the contraction produced by this mixture is potentiated, although the tonic phase is abolished. Depolarization and repolarization are shown by the dotted line. Double vertical lines indicate washing.

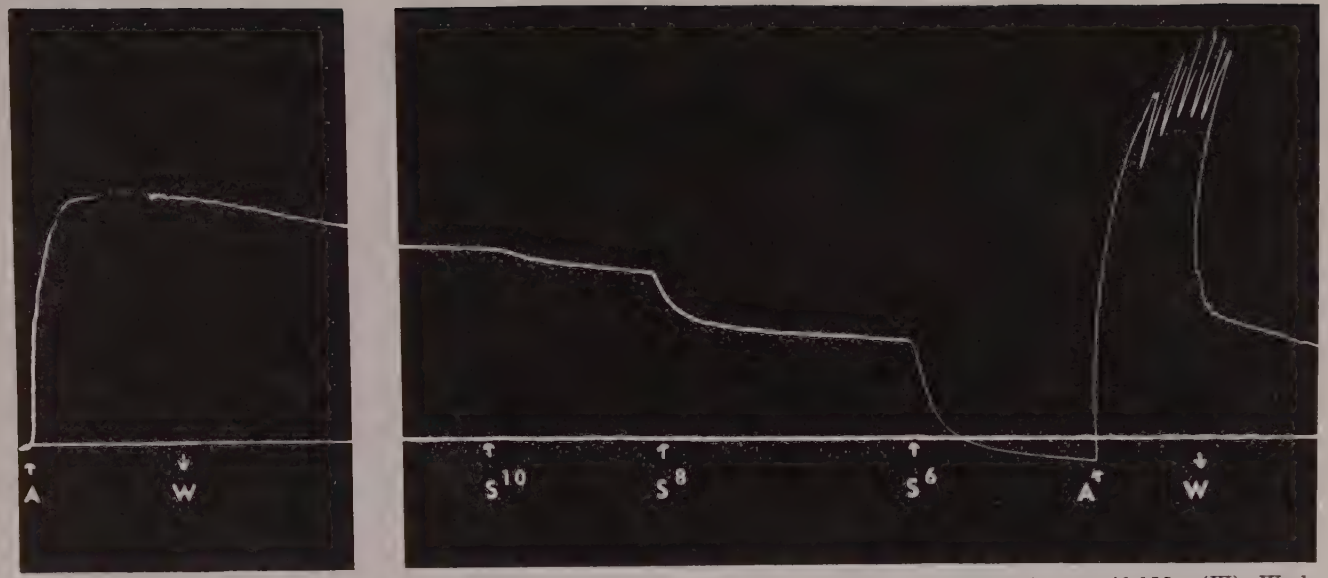

Fig. 5 Relaxation: the interaction of 5-hydroxytryptamine and acetylcholine. (A) Acetylcholine 4×10^{-6} M. (W) Wash. (S^{10}) 5-hydroxytryptamine 10^{-10} M. (S^{8}) 5-hydroxytryptamine 10^{-8} M. (S^{6}) 5-hydroxytryptamine 10^{-6} M. (A) Acetylcholine 4×10^{-6} M. (W) Wash.

10^{-9} M. to 5×10^{-3} M. are applied. Concentrations of 5-hydroxytryptamine as low as 10^{-9} M. produce marked relaxation in a muscle tonically contracted by acetylcholine. The relaxation is approximately proportional to the log of concentration from about 10^{-10} M., below which no relaxation is seen, to 10^{-6} M., where full relaxation occurs. After full relaxation has been effected, responses to acetylcholine are potentiated, as after epinephrine. Prolonged soaking in concentrations as high as 5×10^{-3} M. causes no block of the initial response to acetylcholine, although the tonic phase of contraction is totally abolished above 10^{-6} M. of 5-hydroxytryptamine.

It seemed possible that the potentiation of acetylcholine by epinephrine and by 5-hydroxytryptamine was due to cholinesterase inhibition. High concentrations of epinephrine can inhibit cholinesterase activity (Waelsch and Rackow, '42). However, potentiation of the acetylcholine response by epinephrine and 5-hydroxytryptamine is still seen after eserinization of the byssus retractor and after prolonged soaking in 10^{-5} M. DFP. A few manometric determinations indicated that the hydrolysis of acetylcholine by byssus muscle homogenates is not inhibited by 10^{-3} M. epinephrine.

Determination of the physiological significance of the effects of pharmacological agents

The evidence of Winton ('37) and Fletcher ('37c) indicates that electrical stimulation of the ARBM directly can bring about tonic contraction, phasic contraction or relaxation. It has been shown above that pharmacological agents induce these same responses. To complete the circumstantial evidence that both electrical stimuli and drugs effect identical physiological changes the following results are hereby appended:

1. The tonic contraction following acetylcholine is relaxed by repetitive electrical pulses.

2. The tonic contractions following long cathodal pulses or mechanical stimulation are relaxed by epinephrine.

3. The height of the contractile response to long cathodal pulses is potentiated, and the tonic phase of the contraction abolished by soaking in high concentrations of epinephrine.

On the basis of this and the preceding pharmacological evidence, it is postulated that the effects of application of acetylcholine and of 5-hydroxytryptamine, do in fact represent physiological events concerned in the transmission respectively of excitatory and of inhibitory impulses in this muscle. This postulate is strongly supported by evidence which indicates that the ARBM normally contains both acetylcholine and an active acetylcholinesterase as well as the inhibitory substance 5-hydroxytryptamine.

Acetylcholine content and cholinesterase activity. Two determinations on the Venus heart showed an inhibitory effect equivalent respectively to 1.2 and 1.1 μg acetylcholine per gram wet weight of tissue. A control assay, using homogenate in which the muscle cholinesterase was still active, showed considerably less inhibition. The inhibitory effect could be totally abolished by adding active serum cholinesterase to the homogenate before the test, or by pretreating the Venus heart with Mytolon, an acetylcholine blocking agent.

The manometric study of cholinesterase activity revealed a QChE of 0.167 mg acetylcholine hydrolyzed by 100 mg tissue (wet weight) per hour. This is an average of 5 experiments with a maximum deviation from the average of 0.014 or approximately 8%. In a series of 4 experiments under identical conditions, neither benzoylcholine nor acetyl-β-methylcholine was hydrolyzed. The enzyme appears to be rather specific for acetylcholine. It resembles some of the marine invertebrate cholinesterases considered by Augustinsson ('46).

Direct evidence of acetylcholine release during activity would be desirable, although not necessary, as additional confirmation of the physiological significance of the acetylcholine response. It has proved impossible to detect such release by methods thus far attempted. It appears certain that less than 0.01 μg acetylcholine is released per gram tissue during maximal electrical stimulation, if there is, in fact, any release at all.

Epinephrine and 5-hydroxytryptamine. Muscle homogenates assayed on a Straub heart contained a substance which augmented the amplitude of the beat but did not characteristically increase the rate, as did equivalent doses of epinephrine. The activity of the homogenate was not diminished by boiling for 10 minutes, or by treatment with hydrogen peroxide.

$Al(OH)_3$ precipitated by neutralization from the acidified homogenate should selectively adsorb epinephrine and related catechol compounds (von Euler, '48). The excitatory action of the homogenate was not appreciably altered after filtering off this precipitate. These facts have been taken as a clear indication that the active substance is not epinephrine nor any closely allied catechol derivative.

The potency and step-wise mode of action of 5-hydroxytryptamine indicated that it might well occur naturally in the muscle tissue. This has proved true. The activity of extracts assayed against known quantities of 5-hydroxytryptamine on the Venus heart, and the estrous rat uterus proved equivalent respectively to 1.0 μg 5-hydroxytryptamine per gram wet tissue and 0.75 μg per gram. 5-Hydroxytryptamine and the extract here proved excitatory. Applied to the byssus retractor, the extract, like 5-hydroxytryptamine, causes relaxation. The equivalent doses cannot be so closely quantitated but likewise show a 5-hydroxytryptamine content of approximately one μg per gram wet tissue. Erspamer and Ghiretti ('51) failed to detect "enteramine" in extracts of the musculature of *Mytilus galloprovincialis.* It may indeed be totally absent in that species. However, it is possible that it is present in quantities too minute to be detected by the methods employed, or that the presence of such substances as acetylcholine in the extract interfered with detection.

DISCUSSION AND CONCLUSIONS

Problems of structure and innervation

The length of individual muscle fibers. It is assumed that an accurate indication of changes in the membrane potential

of individual muscle fibers can be gained by observation of the demarkation potential between an intact and a depolarized segment of a whole muscle when, and only when, the fibers in question do not end between the recording electrodes. In experiments of long duration, the muscle must in addition be long enough and so arranged that depolarization of one segment does not seriously affect the properties of the intact segment. This last requirement is reasonably satisfied by using muscles of 3 to 4 cm length in the chamber described above. There is evidence that the individual fibers of the ARBM extend the length of the muscle from origin to insertion. Fletcher ('37a) concluded from the results of teasing both fresh and macerated muscles and on the basis of the electrical properties he observed, that the individual fibers run the length of the muscle. Van Nieuwenhoven ('46) confirmed Fletcher's results with macerated preparations and pointed out that the absence in this muscle of certain mechanical properties which Jordan ('38) had observed in short-fibered smooth muscles could best be explained by concluding that the individual fibers extend from origin to insertion. Teasing of fresh and macerated muscles has convinced this author that the single fibers are as long as the muscle itself.

Multiple innervation. The question of multiple innervation is especially pertinent in this muscle, which is capable of two distinct types of contraction and in which a maintained contraction can be rapidly relaxed by appropriate stimuli. Pumphrey ('38) found physiological evidence of dual excitatory innervation of the anterior adductor of Mya; Pawlow (1885) and Benson, Hays and Lewis ('42) demonstrated double (excitor and inhibitor) innervation of the adductor muscles of Anodonta and Pecten respectively. Van Nieuwenhoven ('46) reported that the byssus retractor muscle contracts tonically on "strong" electrical stimulation of the pedal ganglion, and relaxes after "weak" stimulation.

Unfortunately, the author has not yet succeeded in tracing the innervation by histological methods. Part, at least, of the excitatory innervation has been traced from the pedal

ganglion by methylene blue staining and by electrical stimulation. However, Van Nieuwenhoven's results, and the evidence for inhibitory innervation in other closely related bivalve molluscs are taken by this author to justify the working hypothesis that the anterior byssus retractor muscle receives inhibitory as well as excitatory innervation.

The site of action of pharmacological agents. The pedal and cerebral ganglia are removed in dissection and both Fletcher ('37a) and the present author found histologically that there are no intramuscular ganglion cells. Thus, the drugs may exert their effects indirectly through the finer nerve branches and nervous terminations, directly at sites on the muscle "membrane" or directly, in some instances, on the contractile elements. By analogy with non-innervated smooth muscle (as in the amnionic and placental vessels) and chronically denervated smooth muscles, it seems probable that these agents act directly at sites on the muscle fiber itself.

Excitation

Depolarization and contraction. Fletcher ('37a) found that an action potential precedes all contractile responses of the ARBM to electrical stimuli. The present study indicates that contractile responses to KCl, acetylcholine, and epinephrine are invariably associated with depolarization. It is reported elsewhere (Twarog, '52) that other agents which evoke contraction, cause depolarization. The extent of depolarization is always roughly proportional to the tension developed. Eserine and certain other drugs potentiate both depolarization and contraction. Studies of the effects of acetylcholine blocking agents have shown that any block of the mechanical response is associated with a corresponding reduction in the extent of depolarization, just as in the frog rectus abdominis preparation of Fleckenstein, Wagner and Göggel ('50).

The causal relationship of depolarization and contraction cannot be established from the data assembled here. It is certain that with all pharmacological agents studied, contraction never occurs in this muscle in the absence of depolarization, and that both depolarization and contraction are invariably potentiated or blocked in corresponding degree by a given agent. Thus it is probable that depolarization represents an excitatory process leading to tension development. This is further supported by the fact that at threshold concentrations of an excitatory agent, depolarization occurs before a mechanical response can be detected.

The acetylcholine response. The sensitivity of this muscle to acetylcholine and the potentiating action of anticholinesterases pointed to the existence of a cholinergic system. The muscle was found to contain quantities of acetylcholine comparable to mammalian brain and cervical sympathetic ganglia. Cholinesterase activity could be determined. Excitation in the ARBM is apparently mediated by a cholinergic system.

Relaxation: Inhibition of tonic contraction

Membrane polarization and relaxation. Relaxation of the acetylcholine contraction bears no obvious relation to the rate of repolarization. Repolarization appears to be governed by the rate of removal of the depolarizing agent. As evidence, contrast the rapid repolarization after acetylcholine when the muscle cholinesterase is active and the slow exponential course of repolarization in an eserinized muscle. Relaxation is not a function of the rate of removal of the depolarizing agent, as is particularly shown by the failure of high concentrations of acetylcholine blocking agents to hasten relaxation once acetylcholine has acted. In view of these facts, it is not surprising that pharmacological agents which relax the prolonged tonic contraction bring about no detectable changes in membrane polarization, or that Fletcher ('37c) found that tetanic electrical stimulation can produce relaxation even after $MgCl_2$ has abolished the action potential.

The "ratchet" mechanism: Tonus. There is every reason to conclude that this smooth muscle shows true tonus in the sense of a "ratchet" or "catch" mechanism. Continued depolarization, that is, excitation, is not required for maintenance of the contraction. It is, in addition, obvious that the muscle is capable of immediate relaxation upon appropriate stimulation.

The relaxation mechanism. Fletcher ('37c) suggested that the existence of a latent period between tetanic stimulation and relaxation, as well as the fact that there is a strength-duration curve for the relaxing effect, indicate that the electrical stimulus acts through an intermediate process, perhaps the stimulation of inhibitory nerves as in the chromatophore preparations of Bozler ('28). Additional reasons have been cited above for supposing that this process involves inhibitory nerves. If, as appears the case, it is true that the inhibitory process has no electrical sign, there is a striking analogy here to inhibition at the crustacean neuromuscular junction.

From this study emerged an interesting clue to the mechanism of relaxation. The high concentrations of epinephrine needed to produce relaxation, and repeated failure to detect epinephrine in the muscle led to the conclusion that the relaxing effect of epinephrine is primarily of pharmacological interest. The intermediate process could, however, involve the release and action of an epinephrine-like substance. Evidence cited above implicates 5-hydroxytryptamine.

5-Hydroxytryptamine is of widespread natural occurrence. It appears to be identical with enteramine, a substance detected in the posterior salivary glands of *Octopus vulgaris* and *Eledone moschata* by Vialli and Erspamer ('40), in mammalian gastrointestinal mucosa and spleen by Erspamer ('40), the intestine of *Ciona intestinalis* and *Tethium plicatum* by Erspamer ('46) and in the skin of amphibia by Erspamer and Vialli ('51). The chemical identity of the enteraminic substances in these extracts has been proved beyond doubt by Erspamer and Boretti ('51). It has been

identified by Erspamer and Asero ('51) as 5-hydroxytryptamine. Rapport ('49) suggested and Speeter, Heinzelmann and Weisblat ('51) confirmed the suggestion that the vasoconstrictor principle first isolated from beef serum by Rapport, Green and Page ('48) is the creatinine sulfate salt of 5-hydroxytryptamine. Taylor, Page and Corcoran ('51) reported the possible release of serotonin or a very similar substance from brain tissue.

Erspamer and Ghiretti ('51) and Erspamer ('52) described the action of "enteramine" on the molluscan heart and on mammalian blood pressure and smooth muscle organs. Page ('52), and Freyburger et al. ('52) have described effects of 5-hydroxytryptamine on blood pressure and on isolated smooth muscle. 5-Hydroxytryptamine excites the molluscan heart and in many instances acts as a smooth muscle stimulant. An inhibitory action by 5-hydroxytryptamine has been found only in the byssus retractor preparation, but Erspamer ('52), Page ('52) and Freyburger et al. ('52) have found that it is by no means a purely pressor or constrictor substance.

The question of the mechanism of action of 5-hydroxytryptamine remains unanswered. It may be that it acts only upon the contractile elements. Failure to detect membrane changes during relaxation argues for this. The argument is complicated by the striking potentiation by relaxing agents such as epinephrine and 5-hydroxytryptamine of both initial acetylcholine depolarization and contraction. This potentiation invariably accompanies the abolition of the tonic phase of contraction, and is not a result of cholinesterase inactivation. It may well be that previously unavailable (tonically contracted?) "units" are "freed" for action by these agents. Obviously, they are "freed" for electrical as well as mechanical action. It is possible that restoration of full excitability in a tonically-contracted muscle is not reflected directly in the demarkation potential and that these relaxing agents do in fact act wholly or in part through the excitatory system,

fulfilling the linked functions of restoring excitability and of relaxing tonic contraction.

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SUMMARY

1. The demarkation potential measured between an intact and a KCl-depolarized segment of the long-fibered anterior retractor byssus muscle of *Mytilus edulis* accurately reflects membrane polarization. Frequent measurements were made of the demarkation potential during and after application of pharmacological agents to the intact segment. At the same time, mechanical responses were recorded.

2. The course of depolarization in isotonic KCl is described.

3. Acetylcholine ($\geqq 10^{-6}$ M.) causes depolarization and tonic contraction. On washing off the acetylcholine, the muscle immediately repolarizes but the contraction persists. The depolarization and contraction produced by acetylcholine are potentiated by eserine and blocked by certain acetylcholine blocking agents.

4. Acetylcholine is present in the ARBM, as determined by bioassay; so also is an active cholinesterase, as determined manometrically. The excitatory system of this muscle appears to be cholinergic.

5. 5-Hydroxytryptamine (= enteramine, = serotonin) ($\geqq 10^{-9}$ M.) causes prompt relaxation of tonic contractions produced by acetylcholine, cathodal pulses or mechanical stimulation, without causing any measurable change in membrane polarization. High concentrations of 5-hydroxytryptamine actually potentiate the initial depolarization and contraction due to acetylcholine, but the tonic phase of the contraction,

which persists after removal of acetylcholine, is often totally abolished by concentrations as low as 10^{-7} M. 5-hydroxytryptamine.

6. 5-Hydroxytryptamine is present in the ARBM, as determined by bioassay. It is suggested that 5-hydroxytryptamine is of physiological significance as an integral part of the inhibitory system of this muscle.

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